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13. ABSTRACT *(Maximum 200*

We have identified in a range of human breast cancer biopsy samples, several known and unknown estrogen receptor (ER) variant mRNAs as well as some ER mRNAs containing inserted nucleotide sequences. The variant ER mRNAs are likely generated by alternative splicing mechanisms, while the inserted ER mRNAs are likely to be generated from a mutated ER allele in the tumor cells. All types of variant ER mRNAs so far identified, could be detected in normal human mammary tissue. The expression of some of these variant ER mRNAs was significantly higher in breast tumors than in normal breast tissue. Moreover, the pattern of ER variant mRNA expression changes in breast tumors and breast cancer cells with characteristics of poor prognosis and lack of sensitivity to endocrine therapies. Our data suggest that the mechanisms generating ER variant mRNAs exist in normal breast tissue and may be deregulated in breast cancer tissues. Further, the data support and are consistent with our working hypothesis that the expression and/or altered expression of variant/abnormal ER in human breast cancer is one mechanism associated with the development of endocrine resistance and the progression from hormone dependence to independence. We have also generated data consistent with the ability of some ER variant mRNAs to be stably translated in vivo in some human breast cancers.

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5. INTRODUCTION:

The goal of this research is to address the role of variant/abnormal estrogen receptor (ER) expression in the progression of human breast cancers from hormone dependence to independence. The progression of breast cancer from hormone dependence to independence is a clinically significant problem since it limits the effectiveness of the relatively non-toxic hormonal therapies such as antiestrogens and progestins[1]. The hormone-dependent phenotype is characterized by the presence of ER in the breast tumor, but only 50% of receptor positive breast tumors respond to endocrine therapies and of those which initially respond a significant proportion will eventually develop resistance to these therapies. Furthermore, the development of resistance to endocrine therapy occurs despite the continued expression of ER in the tumor, in at least 50% of cases. It is the molecular mechanisms of this form of resistance i.e. the steroid receptor positive/hormone resistant human breast tumors, that this research proposal addresses. Elucidation of these mechanisms will provide information necessary either to prevent the occurrence of hormone resistance, reverse it or develop new treatments for the resistant tumors. As well, novel treatment response markers in human breast tumors are likely to be identified. Although multiple mechanisms are likely to be involved in hormone resistance and progression to hormone independence[1] in human breast cancer, this grant proposal focuses on one possible mechanism: the involvement of variant and/or abnormal forms of ER.

The hypothesis to be tested is that the expression and/or altered expression of variant/abnormal ER in human breast cancer is one mechanism associated with the development of endocrine resistance and the progression from hormone dependence to independence.

The specific aims to address this hypothesis are:

1. To systematically investigate alterations that occur in the ER mRNA in human breast cancers.
2. To characterize structurally and functionally those abnormal ER mRNAs occurring most frequently and determine their involvement in the development of hormone independence and progression in HBC.
3. To develop specific tools to investigate the expression of the corresponding proteins that may be translated from altered or variant ER mRNAs.
4. To assess the biological significance of ER variants in human breast cancer by determining the relationship between the level of expression of ER variants in human breast

cancer biopsies and the expression of the normal ER, known estrogen responsive genes, histopathological parameters and other known prognostic factors .

6. BODY:

The **first task** in the "Statement of Work" was to systematically investigate alterations that occur in the estrogen receptor (ER) mRNA in human breast tumors. Figure 1 (in reference 2, see appendix 1) shows the approach taken to investigate alterations in the E-domain of the human ER mRNA. Reverse transcription and polymerase chain reaction amplication (RT-PCR) was carried out on RNA isolated from 212 individual human breast tumors. Twenty ul of each PCR reaction were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Using these criteria altered sized RT-PCR products could be detected in some human breast biopsy samples. Using primer set 1, a major 483 bp fragment was detected in 86 % of the breast tumors analyzed (Figure 2 in reference 2, see appendix 1). This fragment is the expected RT-PCR product for the normal, wild-type ER mRNA. Fourteen percent of tumors contained little or no detectable ethidium bromide staining bands, and were classified as ER mRNA negative. A faint 344 bp band was detected after ethidium bromide staining in 9% of the tumors analyzed (see 4th and 7th samples in second panel Figure 2, in reference 2, see appendix 1). This band is the size expected for an RT-PCR product derived from a previously identified exon 5 deleted-ER transcript[3]. Southern blotting and hybridization with radiolabelled human ER cDNA indicated that both the 483 bp and the 344 bp PCR products specifically hybridized with human ER cDNA (Figure 3, in reference 2, see appendix 1). Further, Southern blotting and hybridization with radiolabelled human ER cDNA enhanced the frequency with which the exon 5 deleted ER transcript was detected (see Figure 3 in reference 2, see appendix 1), from 9 to 30%. A previously identified exon 7 deleted ER mRNA[4] was also identified in all tumors which expressed the wild-type ER mRNA (Figure 4 in reference 2, see appendix 1).

Interestingly, in several breast tumor samples an RT-PCR product larger than that expected for the wild-type ER mRNA was clearly detected by ethidium bromide staining (Figure 2 in reference 2, see appendix 1). These transcripts were identified as larger than wild-type ER mRNA RT-PCR products in 9.4% of 212 human breast tumors analyzed. The data suggest nucleotide insertions are present in ER mRNA of some breast tumors. Cloning and sequencing of the larger RT-PCR products identified three different types: a complete duplication of exon 6 occurring in 7.5 % of tumors; a complete duplication of both exons 3 and 4 occurring in 1 tumor; and a 69 nucleotide insertion between exons 5 and 6 occurring in 3 tumors . Open reading frame analysis suggested that exon 6 duplicated transcripts encoded a 51.4 kDa ER-like protein truncated just after exon 6 sequences; the

exon 3 and 4 duplicated transcript encoded a 83.3 kDa protein containing duplication of ER amino acid residues encoded by exons 3 and 4; the 69 nucleotide insertion was inframe, adding 23 novel amino acid residues between residues 412 and 413 of the normal ER protein to produce a 68.8 kDa protein (Figure 8 in reference 2, see appendix 1). It is unknown if these novel ER-like mRNAs are stably translated *in vivo*. Any resulting protein would be structurally altered, however, possibly resulting in altered function.

Our accumulated data suggest that while the generation of the exon deleted ER variant mRNAs and the truncated clone 4 type ER variant mRNA[5] is likely to occur via an alternative splicing mechanism[6] the generation of the inserted and exon-duplicated ER mRNAs is likely to occur due to a mutant ER allele in the breast tumor cells[7]. The 69 nucleotide sequence which is found inserted between exon 5 and 6 of the wild-type ER mRNA was found normally present in intron 5 of the wild-type ER gene[7]. Furthermore, a point mutation was identified in intron 5 of the ER gene isolated from the breast tumor from which the 69 nucleotide inserted ER mRNA was first isolated and characterized. The mutation results in the generation of a consensus splice donor sequence immediately 3' to the 69 nucleotide sequence, and there is a consensus splice acceptor sequence normally present immediately 5' of the 69 nucleotide sequence[7]. We hypothesize that this enables the 69 nucleotide sequence to be seen as an exon in the mutated allele, therefore resulting in the 69 nucleotide inserted ER mRNA.

RT-PCR analysis of other regions of the ER mRNA (Figure 1A in reference 4, see appendix 3) identified previously known ER variant mRNAs deleted in exon 2, exon 3 and a novel exon 2-3 deleted variant[8]. Since several different exon deleted ER variant mRNAs are expressed in any one tumor, we now need to consider all exon deleted variants in any one tumor. Therefore, we have developed a competitive RT-PCR approach using primers at each end of the ER coding region, in order to examine the relationship of total ER deleted variants to expression of the wild type ER, known estrogen responsive genes, histopathological parameters and other known prognostic factors[9] (**Tasks 2 and 5** of our 'Statement of Work' (SOW)). In this approach (illustrated in Figure 1, reference 9, appendix 4), a competitive amplification occurs amongst all exon deleted or inserted ER variant transcripts, which depends on their initial relative expression, and the detection of bands corresponding to specific ER variants reflects the relative expression of these ER variant mRNA species within the sample. A survey of 100 breast tumors[9], showed that the most frequently expressed ER variants at a relatively high abundance were the exon 7 deleted variant, the exon 4 deleted variant, a variant deleted in both exons 3 and 4, a variant deleted in exons 2, 3 and 7, a variant deleted in both exons 4 and 7, a variant deleted in exons 2, 3 and 4, and a variant deleted from within exon 3 to within exon 7[9]. Neither the

exon 5 deleted nor the exon 3 deleted ER mRNAs were detected using this approach. Interestingly, preferential detection of some deleted variants was found to be associated with known prognostic markers in breast cancer[9] (**Task 5**).

Task 2 in the SOW was to develop a quantitative RT-PCR approach for measuring selected ER mRNA variants in RNA isolated from microdissected regions of human breast tissue[10]. This approach was necessary to undertake **task 5** of the SOW, which is to assess the biological significance of ER variant mRNA in human breast cancer progression. Several different assays were developed[8,9,10,11,12,13], each with different advantages and limitations. The type of assay used, was dictated by the the question asked and practical issues.

Using assays developed under **task 2**, we have identified several variant ER mRNA in normal human mammary tissues [8,11,12] (**Task 5**). These include the clone 4 ER truncated variant and variants deleted in exon 2, exon 3, exons 2-3, exon 5 or exon 7. The next question addressed was: Is the level of expression of any of these variants different between normal and neoplastic breast tissue? (**Task 5**). A semi-quantitiative competitive RT-PCR approach was used to determine the relative expression of exon 5- and exon 7-deleted variants to the wild-type ER mRNA in nine normal breast tissues and 19 ER positive breast tumor tissues. The expression of exon 5-deleted ER variant relative to the wild-type ER mRNA was significantly lower ($P < 0.001$) in normal tissue than in tumor tissue (Figure 4 in reference 8, see appendix 3). A similar trend was noted for expression of the exon-7-deleted ER variant mRNA (Figure 4 in reference 8, see appendix 3), but the difference did not achieve statistical significance ($P = 0.476$).

In order to address the question of the level of expression of the truncated clone 4 ER variant in normal and neoplastic tissue (**Task 5**), an RT-triple primer-PCR approach was developed[11] (**Task 2**). The relative level of expression of clone 4 mRNA to the wild-type ER mRNA was compared in frozen sections of normal human breast tissue (8 samples) and human breast tumors with characteristics of good prognosis (10 samples). The expression of clone 4 variant relative to wild-type ER mRNA was significantly lower ($P = 0.03$) in normal tissue than in tumor tissue (Figure 5 in reference 11, see appendix 6). Previously, using an RNAase protection assay, we had determined that the expression of clone 4 variant relative to wild-type ER mRNA was significantly higher in those tumors with characteristics of poor prognosis ($P = 0.0004$) and lack of sensitivity to endocrine therapy ($P = 0.011$)[14]. Interestingly, while addressing the prevalence and 'specificity' of exon deleted mRNA species within the steroid/thyroid/retinoic acid receptor superfamily of genes, we identified, for the first time, the expression of exon deleted progesterone receptor (PR) mRNAs in both normal and neoplastic human breast tissues[15]. Both an exon

6 deleted PR mRNA and an exon 4 deleted PR mRNA were relatively highly expressed and were therefore cloned and sequenced from both normal and neoplastic human breast tissues [15]. Furthermore, the relative expression of the exon 6 deleted PR variant mRNA is significantly higher in human breast tumors than in normal human mammary tissue from unmatched tissue samples[16]. We have not completed studies measuring the relative level of expression of the exon 4 deleted PR mRNA in normal versus neoplastic human breast tissues. Together these data suggest that the mechanisms generating ER and PR variant mRNAs exist in normal breast tissue and may be deregulated in breast cancer tissues. Further, the data support and are consistent with our working hypothesis that the expression and/or altered expression of variant/abnormal ER in human breast cancer is one mechanism associated with the development of endocrine resistance and the progression from hormone dependence to independence.

Task 3 in the SOW has been initiated. Eukaryotic expression vectors for several of the variant and mutant ER mRNAs have been constructed[2,5]. Expression vectors containing the exon 6 duplicated ER cDNA, the exon 3 plus 4 duplicated ER cDNA and the 69 nucleotide inserted ER cDNAs under the control of the SV 40 early promoter in pSG5[17] have been constructed. These constructions also allow for transcription *in vitro. In vitro* translation of the *in vitro* transcribed mRNA produced the expected sized proteins (Figure 8 in reference 2, see appendix 1), and each *in vitro* translated protein was subjected to ligand binding analysis using ^{3}H-estradiol without and with 100 fold excess unlabelled estradiol, to determine non-specific binding as previously described[18]. The exon 6 duplicated ER does not bind estradiol (unpublished data). Both the exon 3 plus 4 duplicated ER and the 69 nucleotide inserted ER bind estradiol but with lower affinity that the wild-type ER (unpublished data). The ability of these proteins to activate transcription of an ERE-tk CAT reporter gene co-transfected transiently into COS-1 (large T-antigen immortalized monkey kidney epithelial cells) and MCF-10 (spontaneously immortalized human mammary epithelial cells) cells was determined in the presence and absence of estradiol (10 nM). The exon 6 duplicated ER and the 69 nucleotide inserted ER had little, if any, detectable transactivation activity under these conditions (unpublished data). The exon 3 plus 4 duplicated ER had estradiol activated transactivation activity which was reduced compared to the wild-type ER (unpublished data). The reduced transactivation activity of these abnormal ERs seemed to be correlated with their reduced binding affinity for estradiol (unpublished data). However, using a "promoter-interference assay" as previously described [19] our preliminary data suggest that the exon 6 duplicated ER, the exon 3 plus 4 duplicated ER and the 69 nucleotide inserted ER can all bind to DNA containing a classical ERE. The ability of each of these abnormal ERs to inhibit the wild-type ER function i.e. to

act as a dominat negative, is being investigated by transient transfection into MCF-7 human breast cancer cells, which express endogenous ER, as well as co-transfection of the wild-type ER and abnormal ER expression vectors into COS-1 and MCF-10 cells. These studies are currently in progress, but no data are as yet available.

As well, clone 4 truncated ER variant cDNA under the control of a CMV promoter has been stably transfected into MCF-7 cells together with the neomycin resistance gene. Several colonies resistant to G418 have been isolated and grown up. Analysis of transgene expression has been initiated. Several isolated colonies transfected with the construct containing the clone 4 cDNA were shown to overexpress the clone 4 transgene mRNA compared to colonies transfected with the vector alone, as determined by Northern blotting (unpublished data, see figure in appendix 13). As well, preliminary data suggest that over-expression of an appropriately sized protein (approximately 24 kDa) which is immunoreactive with an ER antibody (H226) recognizing an epitope in the A/B region of the wild type ER protein, and predicted to be present in the clone 4 truncated ER protein, has been identified in the clone 4 transgene overexpressing colonies (unpublished data).When several independent clones have been identified with overexpression of the transgene, these will be tested, under tissue culture conditions, for their responsiveness to estrogen, monohydroxytamoxifen, ICI 164 384 and progestins. As well the level of expression and estrogen regulation of known estrogen regulated genes such as pS2 and PR, will be determined in the transgene expressing cells versus the vector alone transfected controls. We have developed an ER positive, estrogen independent human breast cancer cell line from an originally estrogen responsive human breast cancer cell line by long term growth in estrogen depleted medium[20]. Interestingly, the estrogen independent cell line was found to overexpress an exon 3 plus 4 deleted ER variant mRNA[21]. We are investigating a possible role of this ER variant in the development of estrogen independence in breast cancer cell models (**Task 5**).

Task 4 has been initiated. Firstly, a synthetic peptide containing the novel 6 amino acids present in the predicted clone 4 ER protein has been ordered from Chiron Inc. This peptide will be conjugated to diptheria toxoid and this antigen will be used to raise antibodies to the clone 4 ER protein. These antibodies will provide us with a specific tool to investigate the expression of the clone 4 ER protein. As well, we have analyzed a group of human breast tumors immunohistochemically for ER expression[22] using antibodies that recognize either an N-terminally localized epitope in the wild-type ER protein (expected to be present in many ER variants), or a C-terminally localized epitope in the wild type ER protein (not expected to be present in many ER variants). It was found that the antibody recognizing the C-terminally localized epitope correlated far better with ligand binding

assays than did the antibody recognizing the N-terminally localized epitope. Furthermore, although in many tumors the immunohistochemical results using each antibody showed good concordance, in some tumors the results were discordant, with the signal tending to be higher more often with the N-terminal antibody[22]. Since many of the proteins predicted from variant ER mRNAs would be truncated at the C-terminus and not contain the epitope recognized by the C-terminal antibody[22], one interpretation of these data would be that truncated variant ER proteins are more highly expressed in the discordant group of tumors.This hypothesis was tested by investigating the pattern and relative expression of variant ER mRNAs in the discordant and concordant groups of breast tumor[23]. We used our previously developed assays[9,11] to determine the relative pattern of expression of exon deleted ER mRNAs and to measure the relative expression of the clone 4 truncated ER mRNA in these two groups of breast tumors. Several ER variant mRNAs which encode putative truncated ERs recognized only by an N-terminal targeted antibody were preferentially and relatively more highly expressed in the discordant group of tumors. These ER variants were: the clone 4 truncated ER mRNA, an exon 2, 3 plus 7 deleted ER mRNA, an exon 2, 3 and 4 deleted ER mRNA and a variant deleted from within exon 3 to within exon 7[23]. The data suggest that the ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER measured by immunodetection methodologies. Further, the data are consistent with the ability of some ER variant mRNAs to be stably translated *in vivo* **(Task 4)**.

7. CONCLUSIONS.

Our data suggest that both variant and mutant ER mRNAs exist in human breast cancers. Further, several different types of variant ER mRNAs can be expressed in any one tumor. Therefore, a need existed to develop methods to consider them as a whole in human breast cancer, in order to examine the relationship of total ER variant expression to expression of the normal ER, known estrogen responsive genes, histopathological parameters and other known prognostic factors. We have developed a competitive RT-PCR approach using primers at each end of the ER coding region, such that a competitive amplification occurs amongst all exon deleted or inserted ER variant transcripts, which depends on their initial relative expression. The detection of bands corresponding to specific ER variants reflects the relative expression of these ER variant mRNA species within the sample. A survey of 100 tumors using this approach demonstrated that the preferential detection of some deleted variants was associated with known prognostic markers in breast cancer.

With respect to investigating the biological significance of individual ER variants in human breast cancer, we have shown that several variants can be detected in normal human

breast tissue. Moreover at least two of these variants are more highly expressed in breast tumors than in normal mammary tissue. We have also shown that the expression of one variant ER mRNA (clone 4 truncated variant) relative to wild-type ER mRNA was significantly higher in those tumors with characteristics of poor prognosis and lack of sensitivity to endocrine therapy. As well we have found that overexpression of a multiple exon deleted variant ER mRNA was found to accompany the development of estrogen independence in a human breast cancer cell line model. We have also generated data which are consistent with the hypothesis that at least some ER variant mRNAs are stably translated *in vivo* in some human breast cancer tissues.

Our data suggest that the mechanisms generating ER variant mRNAs exist in normal breast tissue and may be deregulated in breast cancer tissues. Further, the data support and are consistent with our working hypothesis that the expression and/or altered expression of variant/abnormal ER in human breast cancer is one mechanism associated with the development of endocrine resistance and the progression from hormone dependence to independence.

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APPENDIX 1

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Novel Mutations in the Estrogen Receptor Messenger RNA in Human Breast Cancers*

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ABSTRACT

One mechanism that has been suggested to play a role in the progression of human breast cancer from hormone dependence to independence is the expression or altered expression of mutant and/or variant forms of estrogen receptor (ER). Two major types of variant ER messenger (m)RNA have been identified in human breast biopsy samples so far: truncated transcripts and exon deleted transcripts. In this study we provide data indicating the existence of a novel type of abnormal ER mRNA. These transcripts were identified as larger than wild-type ER mRNA RT-PCR products in 9.4% of 212 human breast tumors analyzed. The data suggest nucleotide insertions are present in ER mRNA of some breast tumors. Cloning and sequencing of the larger RT-PCR products showed three different types: a complete duplication of exon 6 occurring in 7.5% of tumors; a complete duplication of both exons 3 and 4 occurring in 1 tumor; and a 69 nucleotide insertion between exons 5 and 6 occurring in 3 tumors. Open reading frame analysis suggested that exon 6 duplicated transcripts encoded a 51.4 kDa ER-like protein truncated just after exon 6 sequences; the exon 3 and 4 duplicated transcript encoded a 83.3 kDa protein containing duplication of ER amino acid residues encoded by exons 3 and 4; the 69 nucleotide insertion was inframe, adding 23 novel amino acid residues between residues 412 and 413 of the normal ER protein to produce a 68.8 kDa protein. It is unknown if these novel ER-like mRNAs are stably translated *in vivo*. Any resulting protein would be structurally altered, however, possibly resulting in altered function. (*J Clin Endocrinol Metab* **81:** 1420–1427, 1996)

THE NATURAL history of human breast cancer appears to be a progression from hormone dependence to independence and the development of resistance to endocrine therapies (1). This often occurs despite the continued expression of the estrogen receptor (2). It has been suggested that one mechanism associated with this progression is the expression, or altered expression, of mutant and/or variant forms of the estrogen receptor (ER) protein (3). Support for this hypothesis has been derived from studies over the last decade which have identified a role for mutant or variant steroid hormone receptors in other human diseases (4–6).

Several abnormal or variant forms of ER messenger (m)RNA species have been recently identified in some breast cancer cell lines and human breast cancer biopsy samples (7–13). These include truncated transcripts and transcripts containing precise exon deletions that potentially could encode ER-like proteins with altered function (7, 9, 10). Moreover, recent studies using gel-shift/antibody supershift and Western blot analyses suggest that ER-like proteins corresponding to some of the variant ER-like transcripts may exist (14–16). Whether these are the only alterations in the ER to be found in human breast cancers and the exact frequency of occurrence of abnormal ER mRNAs are important unanswered questions. Such information is essential to understanding the significance of and possible involvement of expression of these abnormal/variant ER molecules in the progression of human breast cancer from hormone dependence to independence.

In undertaking a detailed systematic study of the type and frequency of occurrence of abnormal/variant ER mRNAs in human breast tumors we have now identified novel forms of ER-like mRNAs which contain exon duplications or other inserted sequences.

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Materials and Methods

RNA isolation

Total RNA was isolated from human breast cancer biopsy samples as previously described (10, 17). The integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described (17).

RT-PCR

Total RNA (1 μg per reaction), denatured at 65C for 3 min was reverse transcribed in a final volume of 50 μL made 1 × with the reaction buffer supplied with the m-MLV reverse transcriptase (5 ×: 0.25 m Tris-HCl (pH 8.3), 0.375 m KCl, 15 μmol/L $MgCl_2$, 50 mmol/L DTT), using M-MLV reverse transcriptase (200 units/reaction, Gibco/BRL Burlington, Ont.), 0.01 mol/L DTT, and either an ER specific reverse transcription primer (2.5 μg/reaction, 5'-GAA CTG AGC AAG CAA ATG AAT GG-3') or random hexamers (final concentration 0.5 μm, Gibco/BRL). The reaction was allowed to proceed for 60 min at 37C and then was terminated by heating at 90C for 5 min. 1 μL of this reaction was amplified by PCR for 30 cycles of 1 min 94C, 1 min 60C, and 1 min 72C. The reaction volume was 50 μL containing 0.01 mol/L Tris-HCl (pH 8.3), 0.05 mol/L KCl, 2.5 μmol/L $MgCl_2$, 0.2 mg/mL gelatin, 1 mmol/L dNTPs, 1 μL of each primer (200 ng/μL) and 1 unit of Taq Polymerase (Gibco/BRL). 20 μL of the PCR reactions were electrophoresed in agarose gels (1–2% depending on expected sizes of products) and visualized by ethidium bromide staining. The primer sequences are as follows:

1) Set 1: 1 upper (sense) 5′ - CAG GGG TGA AGT GGG GTC TGC TG - 3′ (priming site in exon 4); 1 lower (antisense) 5′ - ATG CGG AAC CGA GAT GAT GTA GC - 3′ (priming site in exon 6)
2) Set 2: 2 upper (sense) 5′ - TCC TGA TGA TTG GTC TCG TCT GG - 3′ (priming site in exon 5); 2 lower (antisense) 5′ - ATG CTC CAT GCC TTT GTT ACT CA - 3′ (priming site across exon 6/exon 7 boundary)
3) Set 4: 4 upper (sense) same as 2 upper; 4 lower (antisense) 5′ - CAG GGA TTA TCT GAA CCG TGT GG - 3′ (priming site in exon 8).
4) Specific primers for exon 6 duplicated transcripts: 6 × 2 upper (sense) 5′ - GCA GGG AGA GGA GTT TGT GTG - 3′; 6 × 2 lower (antisense) 5′ - ATG CGG AAC CGA GAT GAT GTA - 3′
5) Specific primers for exon 3–4 duplicated transcripts: 3–4 × 2 upper (sense) 5′ - ATG GAG TCT GGT CCT GTG AG - 3′; 3–4 × 2 lower (antisense) 5′ - ATA GTC GTT ATG TCC TGG CA - 3′
6) Specific primers for novel inserted sequence between exon 5 and 6: upper (sense) 5′ - TTT GCT CCT AAC TTG CTC TTG - 3′ (priming site in exon 5); lower (antisense) 5′ - AAC TGG AGG AAG TGG AGG TTG - 3′ (priming site in novel sequence).

Southern blot analysis

1–5 μL of PCR products were subjected to Southern blotting as previously described (10), and the resulting blots were hybridized with human ER cDNA (OR-8, 18) labeled with ^{32}P phosphate by nick-translation as previously described (10).

Cloning and sequencing

Abnormal sized PCR products were isolated from low melting point agarose gels (NuSieve GTG, FMC Bioproducts, Rockland, ME) and ligated into the TA cloning vector pCRII, using the TA Cloning Kit (Invitrogen, San Diego, CA). The inserts were sequenced using the Sequenase Kit (USB, Cleveland, OH) or the T_7 Sequencing Kit (Pharmacia, Baie d'Urfe, Quebec).

In vitro transcription translation

Expression vectors for the different exon duplications and novel insertion were constructed by insertion of the appropriate fragment from the TA cloning vector into appropriate restriction sites of HEGO (gift from Dr P Chambon INSERM, Strasbourg, France 19). Sense RNA was transcribed from these construct using T_7 RNA polymerase and translated *in vitro* in the presence of ^{35}S-methionine using rabbit reticulocyte lysate (Promega, Madison, WI). The products were analyzed under reducing conditions by SDS/polyacrylamide (7.5%) gel electrophoresis followed by autoradiography.

Results

Figure 1 shows the approach taken to investigate alterations in the hormone binding E-domain of the human ER

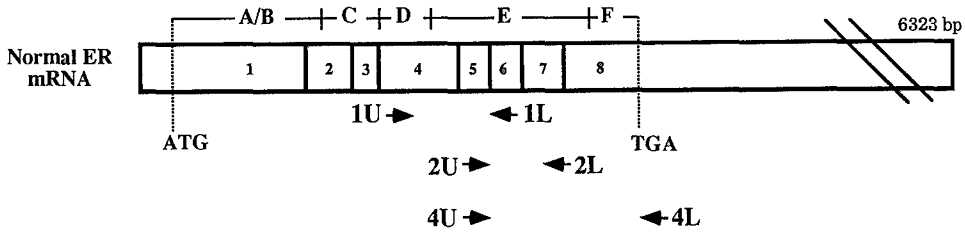

FIG. 1. RT-PCR analysis of the E domain of the human ER mRNA. Schematic diagram of the approach taken to investigate alterations in the E-domain of the human ER mRNA using RT-PCR. U, upper (sense) PCR primer; L, lower (antisense) PCR primer.

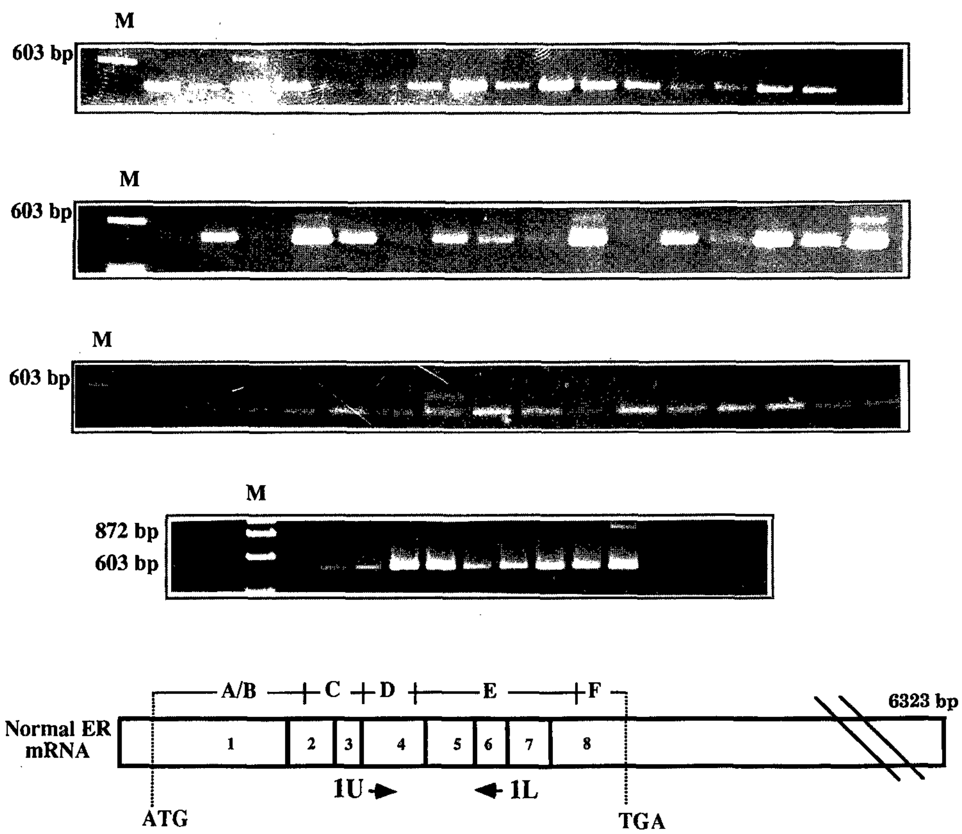

FIG. 2. RT-PCR analysis using primer set 1 of RNA isolated from human breast tumors. RT-PCR products obtained using PCR primer set 1. Twenty μL of the resulting PCR products were size-separated by electrophoresis on agarose gels. The RT-PCR products were visualized by ethidium bromide staining. M, phi X 174 RF DNA/*Hae* III fragments were used as molecular size markers; the 603 and 872 bp fragments are shown on the left hand side. *Bottom panel* shows localiztion of the PCR primers used in this analysis.

mRNA. RT-PCR was carried out on RNA isolated from 212 individual human breast tumors. Twenty μL of each PCR reaction were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Using these criteria altered size RT-PCR products could be detected in some human breast biopsy samples. Using primer set 1, a major 483 bp fragment was detected in 86% of the breast tumors analyzed (Fig. 2). This fragment is the expected RT-PCR product for the normal, wild-type ER mRNA. Fourteen percent of tumors contained little or no detectable ethidium bromide staining bands and were classified as ER mRNA negative. A faint 344 bp band was detected after ethidium bromide staining in 9% of the tumors analyzed (see 4th and 7th samples in *second panel* Fig. 2). This band is the size expected for an RT-PCR product derived from a previously identified exon 5 deleted-ER transcript (9). Southern blotting and hybridization with radiolabeled human ER cDNA indicated that both the 483 bp and the 344 bp PCR products specifically hybridized with human ER cDNA (Fig. 3). Further, Southern blotting and hybridization with radiolabeled human ER cDNA enhanced the frequency with which the exon 5 deleted ER transcript was detected (see Fig. 3) from 9 to 30%.

Interestingly, in several breast tumor samples an RT-PCR product larger than that expected for the wild-type ER mRNA was clearly detected by ethidium bromide staining (Fig. 2). Larger sized RT-PCR products were detected by ethidium bromide staining in 9.4% of the breast tumors analyzed. The first and second panels in Fig. 2 show examples of tumors containing a larger RT-PCR product of approximately 616 bp. *Panel 3* of Fig. 2 shows a tumor in which the larger sized fragment is approximately 552 bp, and *panel 4* of Fig. 2 shows a tumor in which a fragment of approximately 935 bp is detected. Southern blotting and hybridization with a radiolabeled human ER cDNA indicated that all these larger sized RT-PCR products specifically hybridized with human ER cDNA (Fig. 3). The data suggested that these tumors expressed an abundant ER-like transcript containing inserted sequences somewhere within the region bounded by PCR primer set 1. Since the sizes of the larger fragments varied, it was hypothesized that different types of inserted sequences occurred.

The only other set of PCR primers in the E region that detected a fragment other than that expected for the wild-type ER mRNA, was primer set 4. As shown in Fig. 4, the RT-PCR product expected from the wild-type ER mRNA is 668 bp, and this fragment was detected in several tumors. However, an abundant smaller fragment of 484 bp was seen in all cases where the wild-type fragment was detected. This fragment corresponded in size to the previously identified exon 7 deleted ER-like transcript (7). Both PCR fragments hybridized with a radiolabeled human ER cDNA following Southern blotting (see *panel 3* in Fig. 4).

To characterize the apparent insertions in the ER mRNA, the larger RT-PCR products were cloned into TA-cloning vectors and their nucleotide sequence determined. The nucleotide sequence of the 616 bp fragment revealed that this transcript contained an exact exon 6 duplication (Fig. 5A). Sequencing the 935 bp fragment indicated that the transcript

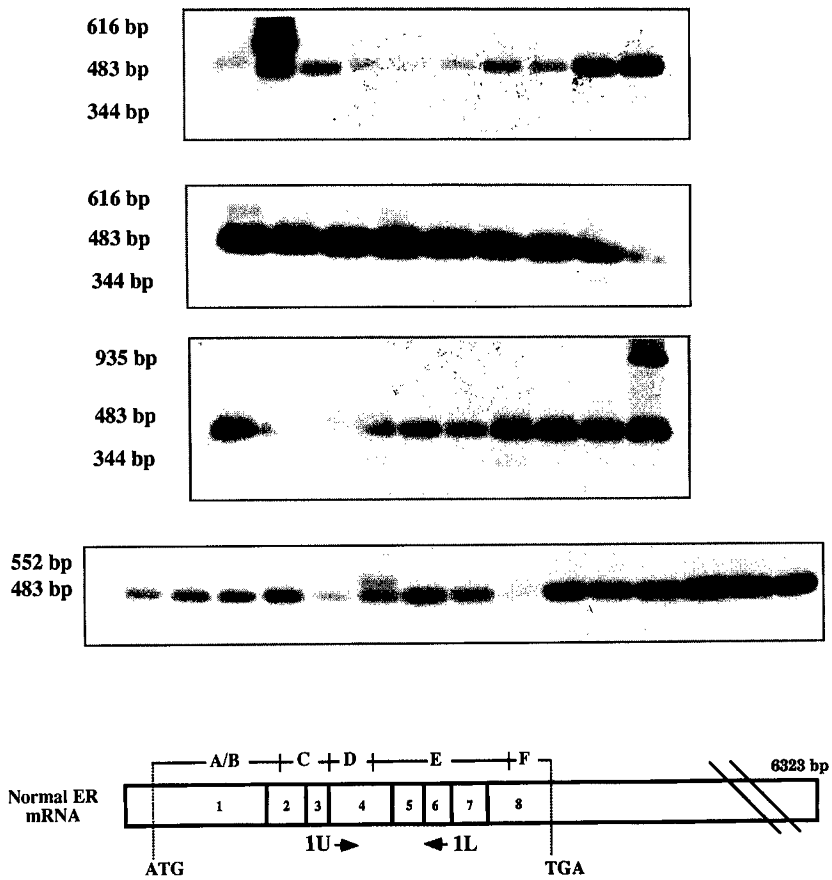

FIG. 3. Specific hybridization of the human ER cDNA with RT-PCR products from human breast cancer samples. A Southern blot of 1–5 μL of RT-PCR products from selected human breast tumors shown in Fig. 2. The DNA was transferred to nitrocellulose membranes and hybridized with radiolabeled human ER cDNA. Estimated molecular sizes of the specifically hybridizing products are shown on the left hand side of the figure. *Bottom panel* shows localization of the PCR primers used in this analysis.

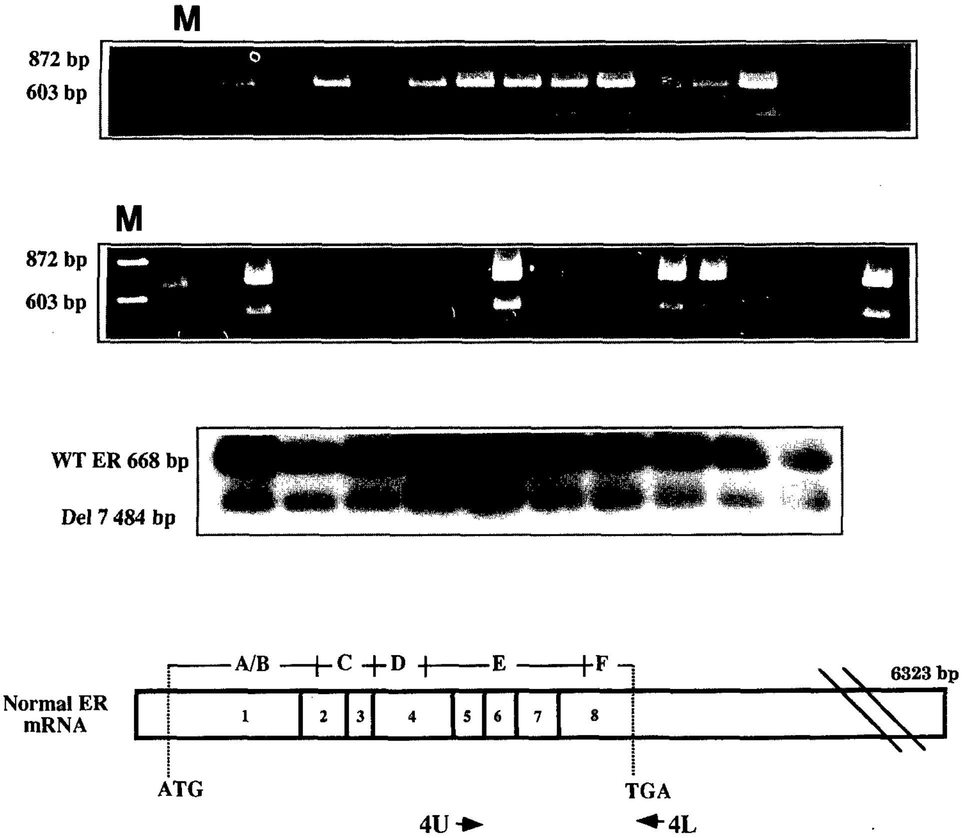

FIG. 4. RT-PCR products obtained using primer set 4 for the human ER mRNA. The top two panels show ethidium bromide stained RT-PCR products from several human breast cancer samples using primer set 4. Twenty μL of RT-PCR products were size-separated by electrophoresis on agarose gels. M, phi X 174 RF DNA/*Hae* III fragments were used as molecular size markers; the 603 and 872 bp fragments are shown on the left hand side. The *third panel* shows a Southern blot of 1–5 μL of RT-PCR products from selected human breast tumors present in the top panels. The DNA was transferred to nitrocellulose membranes and hybridized with radiolabeled human ER cDNA. Estimated molecular sizes of the specifically hybridizing products are shown on the left hand side of the *third panel*. *Bottom panel* shows localization of the PCR primers used in this analysis.

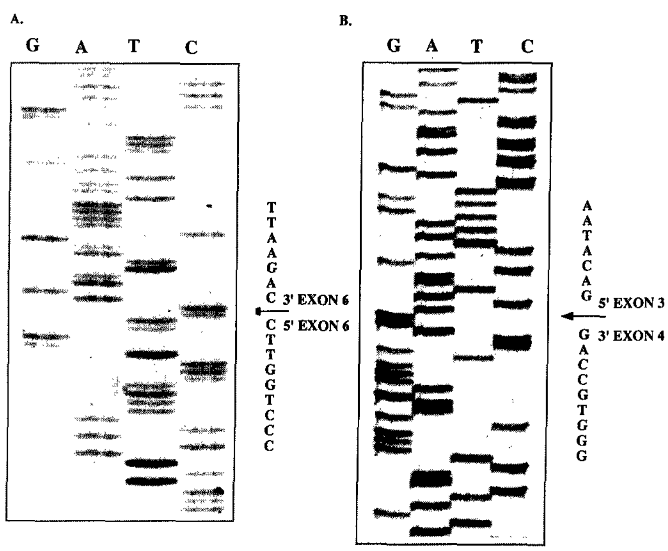

FIG. 5. Nucleotide sequence of exon duplicated ER mRNAs. A. Nucleotide sequence across the novel boundary of the exon 6 duplicated ER-like mRNA. The 616 bp RT-PCR product was cloned into a TA cloning vector and its nucleotide sequence determined. B. Nucleotide sequence across the novel boundary of exon 4/exon 3 ER-like mRNA. The 935 bp RT-PCR product was cloned into a TA cloning vector and its nucleotide sequence determined.

from which it was derived contained exon 4 sequences followed by exon 3 sequences, followed again by exon 4 sequences (Fig. 5B). It was suspected that this latter transcript may have a complete duplication of exons 3 and 4. Using the novel exon4/exon3 boundary sequences found in this transcript a lower (antisense) primer was designed to match an upper (sense) primer located in exon 2 of the human ER. If an ER-like transcript duplicated in exons 3 and 4 was present in the RNA isolated from the tumor in which the original 925 bp product had been detected and cloned (Fig. 6, *panel 1*, lane 7), this primer set (Fig. 6, primer set 34^2 U and L) would result in a 521 bp RT-PCR product. Using these primers, an exon 3 and 4 duplicated ER-like transcript was found at high abundance in only one tumor (Fig. 6, *panel 1*, lane 7).

A similar approach (Fig. 6, primers 6 $\times$ 2 U and L) was used to confirm the exon 6 duplicated ER-like transcript (Fig. 6, *panel 1*, lane 1). Although the expected 443 bp RT-PCR fragment was detected by this approach, other bands were also detected. To further confirm the existence of an exon 6 duplicated ER-like transcript, another set of primers (Fig. 6, 6^2 U and L) was designed that would only detect an exon 6 duplicated transcript. These primers would be unable to amplify the wild-type transcript (Fig. 6, 6^2 U and L), as the primers would face away from each other on the wild-type transcript. However, if an exon 6 duplicated ER-like mRNA was present, a 125 bp fragment would be amplified. The expected 125 bp RT-PCR product was found not only in the tumor from which the exon 6 duplicated transcript was cloned (Fig. 6, *panel 2*, last sample), but also in other breast cancer biopsy samples (*panel 2* in Fig. 6). These data are consistent with the detection of a 616 bp fragment, using set 1 primers, at relatively high abundance in multiple breast cancer samples (see Fig. 2, *panels 1 and 2*). The exon 6 duplicated transcript was detected by ethidium bromide staining in 7.5% of the tumors assayed.

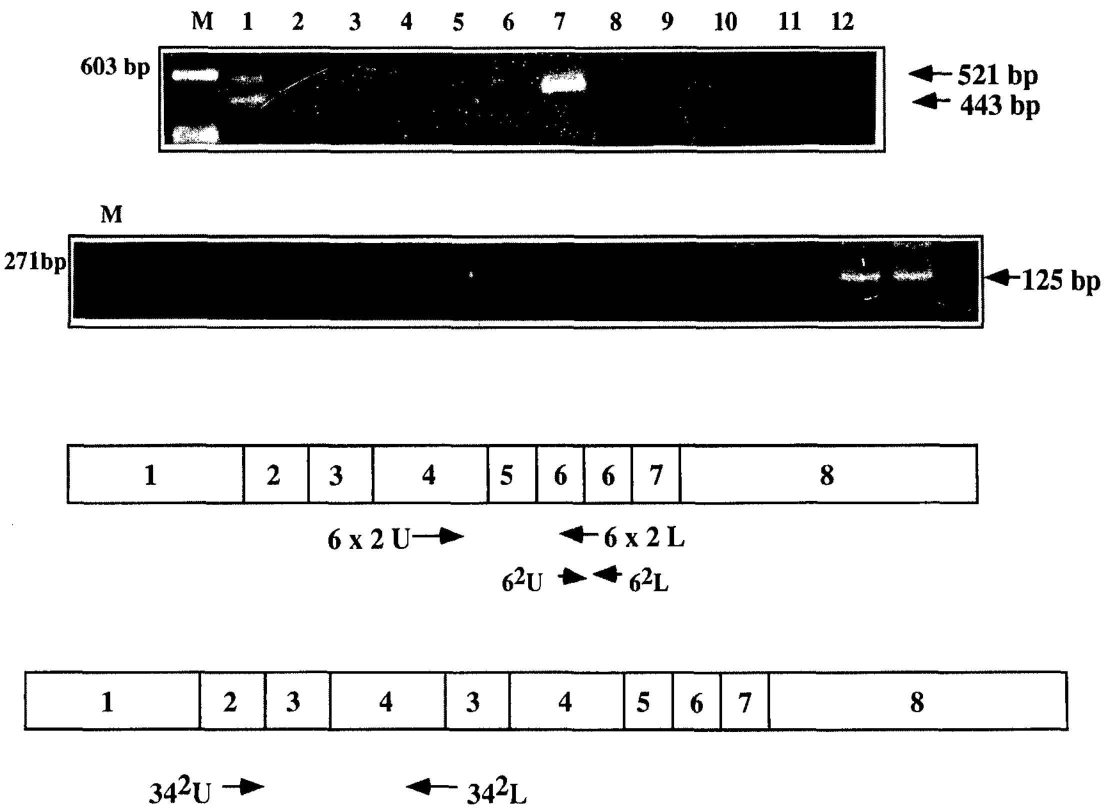

FIG. 6. Specific primers for the exon duplicated ER-like mRNA. The *top panel* (lanes 1–6) shows the RT-PCR products obtained when primer set 6 × 2 was used to specifically detect the exon 6 duplicated ER mRNA. Lane 1 is the tumor from which the exon 6 duplicated ER mRNA was cloned and sequenced. It shows the expected 443 bp product as well as other sized products which were not predicted. Lanes 2–6 are other human breast tumor samples. *Top panel* (lanes 7–12) shows the RT-PCR product obtained when primer set 34^2 was used to specifically detect an exon 3/4 duplicated ER mRNA. Lane 7 is the tumor from which the exon 3/4 duplicated ER mRNA was cloned and sequenced. It shows the expected 521 bp fragment. Lanes 8–12 are other human breast tumor samples. *Middle panel* shows the RT-PCR products from several human breast cancer samples when primer set 6^2 was used to specifically detect the exon 6 duplicated ER mRNA. The last lane is the tumor from which the exon 6 duplicated ER mRNA was cloned and sequenced. It shows the 125 bp expected product, as do several other breast tumor samples. Twenty μL of RT-PCR products were size-separated by electrophoresis on agarose gels and visualized by ethidium bromide staining. Molecular sizes of the RT-PCR products in bp are on the right hand side of the figure. M, phi X 174 RF DNA/*Hae* III fragments were used as molecular size markers; the 603 and 271 bp fragments are shown on the left hand side. *Bottom panels* show localization of the various primers used in this analysis.

Cloning and sequencing of the 552 bp fragment (see *panel* 3 in Fig. 2 and *panel 4* in Fig. 3) identified a novel 69 bp sequence inserted between exons 5 and 6 of the wild-type ER mRNA (Fig. 7). This unique 69 bp sequence was unrelated to the wild-type human ER mRNA. Using the unique 69 bp sequence, primers were designed to detect only ER-like transcripts containing this unique insertion. This 69 bp inserted ER mRNA was detected in two other tumors. In summary, ER-like transcripts containing insertions of one kind or another were detected at relatively high abundance in 9.4% of the tumors assayed. Except for the exon 6 duplicated ER-like transcript the other inserted transcripts were only present at relatively abundant levels in either 1 or 3 individual tumors. Interestingly, such transcripts have not yet been detected in normal human uterus (three individual uteri, data not shown) or normal human normal human mammary tissue (nine individual samples, data not shown).

Fig. 8 shows schematically the predicted open reading frames for each of the three different larger ER-like mRNAs identified in human breast cancer biopsy samples. The exon 6 duplicated transcript contains an inframe stop codon in the second exon 6 sequence, such that a truncated protein of 51.4 kDa is predicted. This protein would be identical to the wild-type ER protein up until amino acid residue 457, followed by 5 additional unique amino acids. The exons 3 and 4 duplicated transcript predicts an open reading frame of 746 amino acid residues, such that the predicted molecular mass of the protein is 83.3 kDa. The predicted protein from the exon 3 and 4 duplicated ER transcript would be identical to the wild-type ER protein up to amino acid residue 366. Thereafter the open reading frame predicts another 380 amino acid residues, which contain a complete duplication of the amino acid residues encoded by exons 3 and 4 followed by the normal wild-type ER amino acid residues encoded by exons 5–8. The unique 69 bp insertion is inframe and codes for 23 novel amino acids inserted between residues 412 and 413 of the normal ER protein. The predicted molecular mass of the protein is 68.8 kDa.

Expression vectors for each of these three different transcripts were constructed, and *in vitro* transcription and translation analyses followed by SDS/PAGE showed that the major protein produced from the exon 6 duplicated tran-

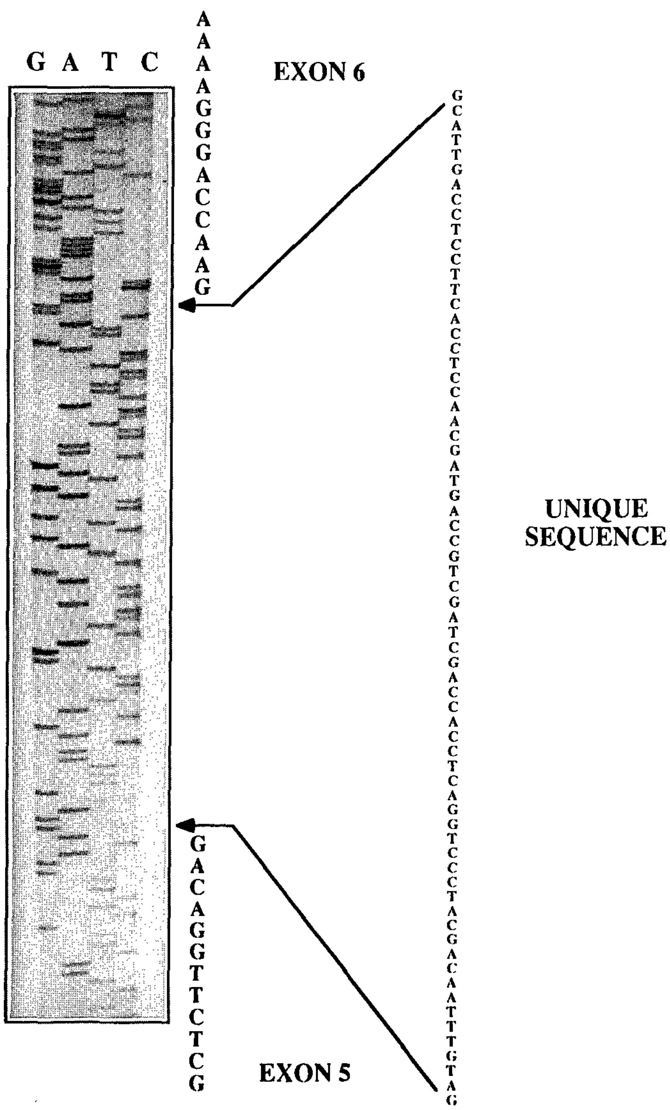

FIG. 7. Nucleotide sequence of the 69 nucleotide insertion between exon 5 and 6 of the human ER mRNA. Nucleotide sequence across the novel boundary of the ER-like mRNA that contained a 69 nucleotide insertion precisely between exons 5 and 6 of the wild-type ER mRNA. The 552 bp RT-PCR product was cloned into a TA cloning vector and its nucleotide sequence determined.

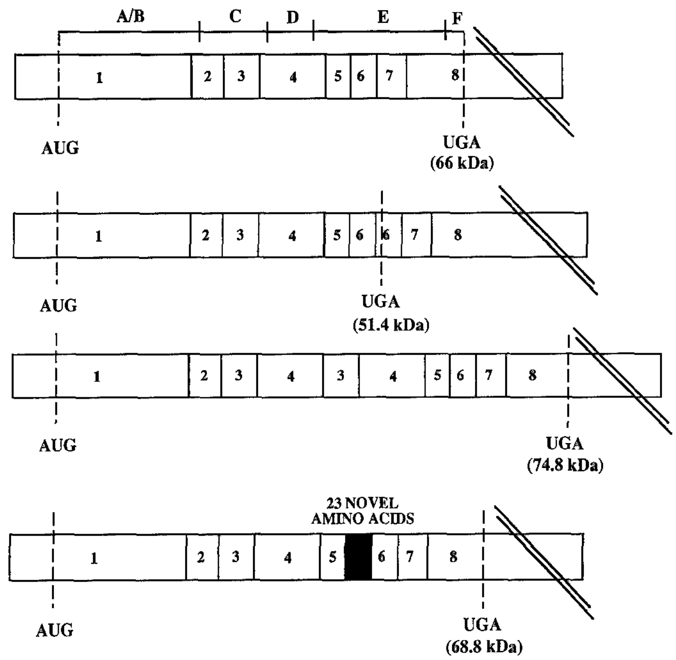

FIG. 8. Open reading frame analysis of the exon duplicated and inserted ER-like mRNAs. Schematic representation of the predicted open reading frames for each of the three different larger ER-like mRNAs identified in human breast cancer biopsy samples.

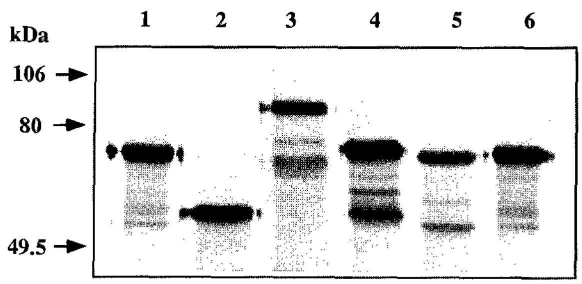

FIG. 9. *In vitro* expression products of the exon duplicated and inserted ER-like mRNAs. *In vitro* transcription and translation analyses of expression vectors for each of the three larger sized ER-like mRNAs. The ^{35}S-labeled proteins were analyzed under reducing conditions by SDS/PAGE (7.5%). An autoradiogram of the results is shown: lane 1, wild-type human ER expression vector obtained from Dr. S. Tsai (Baylor College of Medicine); lane 2, exon 6 duplicated expression vector; lane 3, exon 3/4 duplicated expression vector; lane 4, 69 nucleotide insertion expression vector; lane 5, expression vector for wild-type ER (HEGO, obtained from Dr. P. Chambon, INSERM, Strausberg); lane 6, wild-type human ER expression vector obtained from Dr. S. Tsai (Baylor College of Medicine).

scripts was approximately 51 kDa (Fig. 9); the major protein from the exon 3/4 duplication was approximately 82 kDa (Fig. 9); and the major protein from the unique 69 bp inserted transcript was approximately 69 kDa (Fig. 9).

Discussion

There is a large body of molecular data supporting the potential existence of variant and/or abnormal forms of estrogen receptors in some human breast cancer biopsy samples. Several variant ER-like mRNA molecules have been isolated from some human breast cancer biopsy samples (7–10). The most commonly occurring variant ER-like mRNAs fall into two main groups: 1) the truncated ER-like transcripts (10, 20, 21), which consist of various combinations of exons 1, 2, and 3 of the normal ER mRNA followed by sequences that are not found in the wild-type ER mRNA; and 2) the exon-deleted ER-like transcripts (7, 9, 12, 13), in which precise exon deletions have occurred. ER-like transcripts containing deletions of exon 2, exon 3, exon 4, exon 5, and exon 7 have been reported (7, 9, 12, 13, 22, 23). Simultaneous deletions of both 2 and 3 exons have also recently been reported (22, 23). We now report the occurrence of novel ER-like mRNAs that contain either complete exon duplications or novel sequence insertions. This type of alteration seems to occur in at least 9% of the tumors that we have examined.

The exon duplicated ER-like transcripts appear to be novel

and have not been reported in human breast cancer biopsy samples previously. Interestingly, the identification of an 80 kDa immunoreactive ER-like protein was recently reported in an MCF-7 subclone, that was estrogen independent with respect to growth (24). The transcript possibly corresponding to this protein appeared to contain a precise duplication of both exons 6 and 7 (25). Although we have not identified this exact exon duplication in any of our breast cancer biopsy samples, our data confirm that this type of phenomenon (*i.e.* exon duplication in the ER) can occur *in vivo* and therefore is likely to have some relevance to human breast cancer.

While some small insertions (1–3 nucleotides) have previously been described in the ER mRNA of some breast cancer biopsy samples (26), large insertions such as the 69 nucleotide insertion between exons 5 and 6 have not been previously reported in biopsy samples. Interestingly, an abnormal ER-like transcript was cloned from T-47D_{CO} cells, which contained approximately 130 nucleotide insertions into exon 5 sequences (11). The inserted sequences displayed sequence similarity to the human *alu* family of repetitive sequence (11). The sequence of the 69 nucleotides inserted precisely between exons 5 and exon 6, described in this paper, is not found in the normal wild-type ER mRNA. However, our preliminary data suggest that these sequences can be found in intron 5 of the normal ER gene (Wang M, Dotzlaw H, Fugua SAW, Murphy LC, unpublished data). Interestingly, an abnormal ER mRNA was also cloned from T-47D_{CO} cells that contained approximately 1 kb intron 5 upstream of exon 6 (11).

The truncated clone 4 ER-like mRNA (20) and several of the exon-deleted ER-like mRNAs have now been identified in normal human mammary tissues (22, 23), which suggests that both the truncated and exon-deleted ER-like transcripts are variant and not abnormal products associated with tumorigenesis. However, it seems likely that the levels of some of these transcripts in breast tumors differ from those found in normal mammary tissue (23). The most likely mechanism for the generation of the truncated and the exon-deleted ER-like mRNAs is alternative splicing. However, it is unlikely that an alternative splicing mechanism is responsible for the generation of the ER-like transcripts containing duplicated exons or unique inserted sequences. Further, we have not identified any such ER-like mRNAs in any of the normal mammary or uterine tissues that we have examined so far (23). It is likely, therefore, that the exon-duplicated and inserted ER-like mRNAs are transcribed from an ER gene that has been altered in some human breast cancers. However, the exon-duplicated and inserted ER-like mRNAs were present together with an apparently normal wild-type ER mRNA. We have not found any tumor in which only the inserted or exon-duplicated ER mRNA was present. This suggests that only one allele of the ER gene in these tumors is affected and/or that two populations of tumor cells exist: one in which only the wild-type gene is expressed and one in which the mutated gene is expressed. Interestingly, an MCF-7 sub-line, in which an exon 6 and 7 duplicated ER-like transcript is associated with the expression of an 80 kDa ER-immunoreactive protein, also expresses the wild-type 66 kDa protein (24, 25). These cells have been subjected to several rounds of limiting dilution cloning, and the cells still express both ER-like proteins. Such data suggest that the cells are not a mixed population of cells, *i.e.* one expressing the 66 kDa protein and the other expressing the 80 kDa protein, but that both ER immunoreactive proteins are expressed together in each cell (24). Further, such data provide support for the translation *in vivo* of these mutant ER transcripts.

If the exon 6 duplicated ER-like mRNA were translated *in vivo* the predicted protein would be identical with the wild-type ER protein up until amino acid residue 457 (18) followed by five additional unique amino acids. This predicts for a protein which would contain the A/B and C domains of the wild-type ER but would be truncated in the mid-E domain. Deletion and site-directed mutagenesis data suggest that such a protein would not bind estradiol (27–32). Further, an important dimerization interface and the ligand dependent TAF-2 activity would be missing in the protein predicted from the exon 6 duplicated ER-like mRNA. However, a weaker constitutively active dimerization domain present in the DNA binding domain, as well as the constitutive nuclear localization signal present in exon 4 of the wild-type ER (33) and the ligand independent TAF-1 activity in the A/B domain would still be present (34).

The predicted protein from the exon 3 and 4 duplicated ER transcript would be identical to the wild-type ER protein up to amino acid residue 366. Thereafter, the open reading frame predicts another 380 amino acid residues, which are exactly the same as the wild-type ER protein except that the residues encoded by exons 3 and 4 are completely duplicated. This protein would contain the TAF-1 domain located in the A/B region of the wild-type ER, as well as the DNA binding and dimerization domains, the constitutive nuclear localization signal of the wild-type ER protein, but would then have a third zinc finger encoded by exon 3, another nuclear localization signal followed by the normal E-domain containing ligand binding, TAF-2, and dimerization functions. The presence of the extra ER residues from exons 3 and 4 would likely result in an altered structure of the protein which may affect several of its normal functions.

The unique 69 bp insertion is inframe and codes for 23 novel amino acids inserted between residues 412 and 413 of the normal ER protein. While all residues of the wild-type receptor are present in this protein the inserted sequence may cause an alteration of the structure in the E-domain of this protein, such that some alteration or disruption of function may occur.

It is difficult at this stage to predict the type of altered activity that the above proteins might have, especially because previously identified variant ER mRNAs, such as the exons 5 and 7 deleted ERs, have been shown to encode proteins that, although truncated with respect to the wild-type protein, display marked differences in activities. The exon 5 deleted ER is a constitutively transactivating ER-like protein (7), but the exon 7 deleted ER does not have such activity; however, it has been shown to inhibit the activity of a coexpressed wild-type ER (9) in a dominant negative fashion. Interestingly, this latter activity may be promoter and cell-type specific (9, 35).

In conclusion, we have identified a new type of altered ER-like mRNA expressed at high levels in some human breast cancers *in vivo*. We have grouped them together as

insertion mutants, but within this group two forms exist: precise exon duplications and an insertion of novel sequences not normally found in the wild-type ER mRNA. It is likely that the mechanism by which these transcripts are generated is different from that involved in generation of the truncated and exon-deleted ER transcripts. It is speculated that the expression of these mutant ER-like proteins may alter the ER signal transduction pathway in those tumors which express them, thereby contributing to hormonal progression *in vivo*.

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APPENDIX 2

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A Point Mutation in the Human Estrogen Receptor Gene is Associated with the Expression of an Abnormal Estrogen Receptor mRNA Containing a 69 Novel Nucleotide Insertion.
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A Point Mutation in the Human Estrogen Receptor Gene is associated with the Expression of an Abnormal Estrogen Receptor mRNA containing a 69 Novel Nucleotide Insertion.

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Summary

A novel ER-like mRNA containing a 69 nucleotide insertion precisely between exon 5 and 6 sequences was previously identified in human breast cancer biopsy samples. Data are presented which suggest that the 69 nucleotide sequence is normally present in intron 5 of the human estrogen receptor gene. The region corresponding to and surrounding this 69 nucleotide sequence was cloned and the nucleotide sequence determined. Cloning and sequencing of the corresponding region in genomic DNA isolated from a breast tumor expressing the 69 nucleotide inserted ER mRNA, revealed an A--->G point mutation immediately 3' to the 69 nucleotide sequence. This point mutation resulted in the generation of a consensus splice donor site. A consensus splice acceptor site sequence is normally present immediately 5' to the 69 nucleotide sequence. These data are consistent with the 69 nucleotide sequence being recognized as an exon by the splicing machinery, and resulting in processing of a mature ER mRNA containing the 69 nucleotide insert.

Introduction

We have previously identified in approximately 9% of human breast tumors estrogen receptor (ER)-like mRNAs, which contained inserted sequences [1]. Two types of inserted sequences were identified: one in which complete duplications of normal ER exons were found and one in which 69 novel nucleotides had been inserted between the exons 5 and 6 sequences of the normal ER mRNA. Other altered ER-like mRNAs have also been found in human breast tumors [2]. However, these were mostly truncated ER-like mRNAs [3] or exon deleted ER-like mRNA [4, 5], both of which were most likely generated by some alternative splicing mechanism [6]. It was difficult, however, to suggest an alternative splicing mechanism for either the exon duplicated transcripts or the 69 nucleotide inserted transcript. More likely these transcripts were generated from a mutated ER allele present in some human breast tumors. In this study we present evidence which supports the presence of a mutated ER allele in a breast tumor from which a 69 nucleotide inserted ER-like mRNA was identified and cloned.

Materials and Methods

RNA Isolation: Total RNA was isolated from human breast cancer biopsy samples as previously described [7,8]. The integrity of the RNA was confirmed by denaturing gel electrophoresis [8].

DNA Isolation and Southern Blot Analysis. Southern blot analysis and isolation of genomic DNA from human tumors was as previously described [7]. DNA from bacteriophage clones containing genomic fragments of the human estrogen receptor gene (GHER : gift from Dr P Chambon) [9] was isolated using a plate lysis method [10]. Southern blotting of bacteriophage plaque lifts was carried out according to standard methods [11].

PCR: The thermal profile used was 30 cycles of 1 min at 94^0C, 1 min at 60^0C , and 1 min at 72^0C . The reaction volume was 50 ul containing 0.01 M Tris-HCl (pH 8.3), 0.05 M KCl, 2.5 mM $MgCl_2$, 0.2 mg/ml gelatin, 0.2 mM dNTPs, 1 ul of each primer (200 ng/ul) and 1 unit of Taq DNA Polymerase (Gibco/BRL). 20 ul of the PCR reactions were electrophoresed in agarose gels (1 - 2% depending on expected sizes of products) and visualized by ethidium bromide staining. The primer sequences used are as follows:

Set A: Upper (sense) 5'-TTT GCT CCT AAC TTG CTC TTG - 3' (priming site in exon 5).

Lower (antisense) 5' - CGT AAC TGG AGG AAG TGG - 3' (priming site in novel 69 nucleotides).

Set B: Upper (sense) 5'- TGC CAG TAG CAA CCT CCA CTT - 3' (priming site in novel 69 nucleotides).

Lower (antisense) 5' - CGG AAC CGA GAT GAT GTA GCC - 3' (priming site in exon 6).

Set C: Upper (sense) 5'- TGC CAG TAG CAA CCT CCA CTT - 3' (priming site in novel 69 nucleotides).

Lower (antisense) 5' - CGT AAC TGG AGG AAG TGG - 3' (priming site in novel 69 nucleotides).

Set D: Upper (sense) 5' - CCC AGT CTC AGG TAG GTC TTT - 3' (priming site in intron 5, 5' to novel 69 nucleotides).

Lower (antisense) 5' - GAG TTG GGA AAG CAT AGA GTG - 3' (priming site in intron 5, 3' to novel 69 nucleotides).

Preparation of Radiolabelled Probes. The human ER cDNA (OR-8) [12] was labelled with ^{32}P by nick-translation as described previously [7]. A specific probe for the novel 69 nucleotide sequence was prepared by PCR amplification using primer set C (see above). This primer set generates a 64 bp PCR product which was separated from free nucleotides and primers by low melting point agarose gel electrophoresis (NuSieve GTG Agarose; FMC, Rockland, ME). This product was labelled with ^{32}P-dCTP (0.33 uM final concentration) using 1 PCR cycle under the conditions described above except that the other nucleotides were at a final concentration of 0.33 uM each. The labelled fragment was separated from unincorporated radionucleotides on a Sephadex G-10 column.

Long PCR. This was accomplished using a Perkin Elmer GeneAmp XL Kit (Roche Molecular Systems Inc, Branchburg, NJ) according to the manufacturers instructions.

Cloning and Sequencing: PCR products were isolated from low melting point agarose gels (NuSieve GTG, FMC Bioproducts, Rockland, ME) and ligated into the TA cloning vector, pCR™II using the TA Cloning™Kit (Invitrogen, San Diego, CA). The inserts were sequenced using the T_7 Sequencing Kit (Pharmacia, Baie d'Urfe, Quebec). The region surrounding the 69 nucleotide sequence present in DNA obtained from a tumor expressing the abnormal ER-like transcript was cloned using primer set D.

RESULTS.

We had previously identified 3 human breast tumors which expressed a novel ER mRNA which contained a 69 nucleotide insert precisely between exons 5 and 6 of the wild-type ER mRNA [1]. Due to the precise insertion it was hypothesized that the 69 nucleotide

sequence was normally present in intron 5 of the human estrogen receptor gene and that a mutation in the ER gene of those tumors expressing the abnormal ER mRNA resulted in the 69 nucleotide sequence being recognized as an exon and thus being processed into the mature mRNA. This hypothesis predicts that the 69 nucleotide sequence is present in intron 5 of the normal human ER gene. To address this hypothesis two experiments were undertaken.

Firstly, genomic DNA from T-47D and MCF-7 human breast cancer cells, which do not express the 69 nucleotide inserted abnormal ER mRNA, was isolated and subjected to PCR analysis using specific primers for the 69 nucleotide sequence matched with primers either to sequences in wild-type exon 5 (primer set A) or wild-type exon 6 (primer set B). No PCR products were obtained with primer set B (upper primer specific for the novel 69 nucleotide sequence and lower primer specific for exon 6). However, with primer set A (upper specific for exon 5 and lower specific for the 69 nucleotide sequence), a PCR product of approximately 2.5 kbp was obtained in DNA isolated from both these cell lines (Figure 1).

Secondly, several genomic clones spanning the regions around exon 5 and exon 6 of the human ER (gift from Dr P Chambon) [9] were subjected to Southern blotting of bacteriophage plaques. Duplicate lifts were made. One filter was hybridized with radiolabelled hER cDNA while the duplicate lift was hybridized with a radiolabelled 64 bp probe specific for the novel 69 nucleotide sequence (primer set C). Figure 2 shows the results of this experiment. Human genomic ER clones GHER 9, 10 and 11 all hybridized with the hER cDNA, but only GHER 10 hybridized with the 64 bp probe specific for the novel 69 nucleotide inserted sequence. When DNA isolated from GHER 10 was subjected to long PCR using the primer set A (upper specific for exon 5 and lower specific for the novel 69 sequence), a PCR product of approximately 2.5 kbp was obtained which appeared to be identical in size to that obtained from DNA isolated from the breast cancer cell lines (Figure 1).

Digestion of DNA isolated from GHER 10 with Eco RI yielded four bands. Southern blotting and hybridization with the 64 bp radiolabelled probe specific for the novel 69 nucleotide sequence identified only one band of approximately 3 kbp (data not shown). This band was subcloned into the plasmid Bluescript SK- (Stratagene, La Jolla, CA) and primers specific (sense and antisense) for the novel 69 nucleotide sequence were used individually as sequencing primers. The nucleotide sequence obtained from this analysis confirmed the presence of the novel 69 nucleotide sequence within the clone. As well the sequence of approximately 170 nucleotides either side of the novel 69 nucleotide sequence was also obtained (Figure 3). As shown in Figure 3, the sequence immediately 5' to that of the novel 69 nucleotide sequence, is AG. This is a potential splice acceptor site to partner the splice donor site at the end of exon 5. The sequence immediately 3' of the novel 69 nucleotide sequence is AT. We reasoned that the simplest explanation for the 69 nucleotides being recognized as an exon in the tumor cells expressing the abnormal ER-like transcript, would be a mutation which generated a new splice donor site at the end of the 69 nucleotides which could partner the splice acceptor site preceeding exon 6.

Therefore, DNA was isolated from the human breast tumor biopsy sample originally found to express the 69 nucleotide inserted ER-like mRNA (see Figs 2 & 3 in reference 1). The region surrounding the novel 69 nucleotide sequence was selected and PCR amplified using primers designed from the known normal sequence around this area (Figure 3, primer set D). These primers amplified the expected 252 bp fragment in DNA isolated from non- expressing tumors as well as from the abnormal ER-like mRNA expressing tumor. The 252 bp fragment from the expressing tumor was subcloned and sequenced. Two independent PCRs led to clones some of which when sequenced contained an A--->G mutation immediately 3' to the novel 69 nucleotide sequence (Figure 4). This mutation generates a new splice donor site to partner the splice acceptor site preceeding exon 6. Such a mutation would be consistent with the novel 69 nucleotide

sequence being recognized as an exon and being processed into the mature ER-like mRNA. It should be noted that clones containing the wild-type sequences were also obtained.

DISCUSSION.

Several variant ER-like transcripts have been characterized in human breast cancers [2]. They fall into two main categories: the precise exon deleted transcripts [4, 5] and the truncated transcripts [3, 7] both of which are likely to be generated by an alternative splicing mechanism [6]. More recently we have identified an ER-like transcript which was larger than the wild-type ER mRNA, and indeed cloning and sequencing of this transcript identified a precise insertion of 69 nucleotides between exon 5 and exon 6 sequences [1]. It seemed more likely that this novel transcript was generated from a mutant ER gene, rather than some alternative splicing mechanism. The data presented in this manuscript identify a point mutation in the ER gene present in DNA isolated from the tumor originally found to be expressing high levels of the novel 69 nucleotide inserted ER-like mRNA [1].

The 69 nucleotide sequence was found to be present in DNA obtained from cells containing only the wild-type estrogen receptor gene, at least, as defined by the lack of expression of any detectable abnormal inserted ER-like mRNA. The sequence was further mapped to intron 5 of the human estrogen receptor gene [9]. Sequencing of the region surrounding the 69 nucleotide sequence in the wild-type gene, identified a consensus splice acceptor site immediately 5' to the 69 bp sequence, but not a donor splice site immediately 3' to the 69 bp sequence. When the same region was characterized in DNA isolated from the tumor expressing the abnormal transcript, an A--->G transition was found immediately 3' to the 69 nucleotide sequence which generated a new consensus splice donor site. Indeed the presence of the 69 nucleotide inserted ER-like mRNA in this tumor is consistent with the 69 nucleotides being surrounded with appropriate splicing signals and being recognized as an exon and therefore processed into mature mRNA.

Although wild-type clones were also obtained from the DNA of the tumor expressing the abnormal ER-like mRNA, the A--->G mutation is unlikely to be a PCR

induced error, since the identical mutation was obtained from clones generated from two independent PCR reactions. The mixture of wild-type and mutant is more likely to reflect the presence of both the wild type allele and the mutant allele in the tumor sample. Further, such data are consistent with the original RT-PCR data in which both the wild-type RT-PCR product and the abnormal RT-PCR product were observed in the same RNA extract [1]. It is unclear whether this represents heterozygosity for the mutant allele or alternatively heterogenous cell populations within the tumor sample. Moreover, we cannot exclude that the alteration in this ER allele is germline and not tumor specific, since we have not sequenced the appropriate region in DNA isolated from normal tissue or peripheral blood lymphocytes from the same patient.

In conclusion, our data support the generation of abnormal ER-like mRNA from mutations occurring in the estrogen receptor gene in some human breast cancers.

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LEGENDS TO FIGURES

FIGURE 1

The top panel shows the results of long PCR using primer set A of DNA isolated from GHER 10 bacteriophage containing part of the human estrogen receptor gene [9], and genomic DNA isolated from T47D and MCF-7 human breast cancer cells. Primer set A produced a similar approximately 2.5 kbp fragment as visualized by ethidium bromide staining, from all three DNA samples. Markers are Hind III restriction fragments of lambda phage DNA. The bottom panel shows a schematic representation of the abnormal ER-like transcript with the novel 69 nucleotide insertion (cross-hatched box). The arrows show the approximate positions of primer set A (long PCR) and primer set C (specific probe).

FIGURE 2

Top panels: Bacteriophage containing genomic clones of the regions around exons 5 and 6 of the human estrogen receptor (GHER 9, GHER 10, GHER 11) [9] were subjected to plaque lifts and Southern blotting, using either a human estrogen receptor cDNA (OR-8) [12] or a probe specific for the novel 69 nucleotide insertion (novel sequence probe, see figure 1). The bottom panel shows a schematic representation of the human estrogen receptor cDNA, human estrogen receptor gene and the bacteriophage clones containing various regions of the human estrogen receptor gene [9].

FIGURE 3

Wild-type nucleotide sequence of the region surrounding the 69 nucleotide sequence, which was subcloned from DNA isolated from GHER 10 bacteriophage. The 69 nucleotide sequence is in italics and underlined. The AG immediately upstream of the 69 sequence is bold-faced, as is the AT immediately downstream of the 69 nucleotide sequence. The two bold-faced 21 nucleotide sequences upstream and downstream of the 69 nucleotide sequence are the sequences used to generate PCR primers (primer set D)

which were then used to clone the corresponding region from DNA of a breast tumor expressing the 69 nucleotide inserted ER mRNA .

FIGURE 4

Nucleotide sequence of wild-type and mutant clones obtained from the PCR analysis (primer set D) of DNA from a breast tumor expressing the 69 nucleotide inserted ER mRNA. The arrowheads on the sequencing gel identify the relevant nucleotides in each clone. The asterisk identifies the G point mutation in the mutant clone. Panel A shows representative sequencing gels of both wild-type and mutant clones, and panel B shows the mutant nucleotide sequence, with the 69 nucleotides in italics and underlined and the G mutation bold-faced.

Fig 1

Figure 1

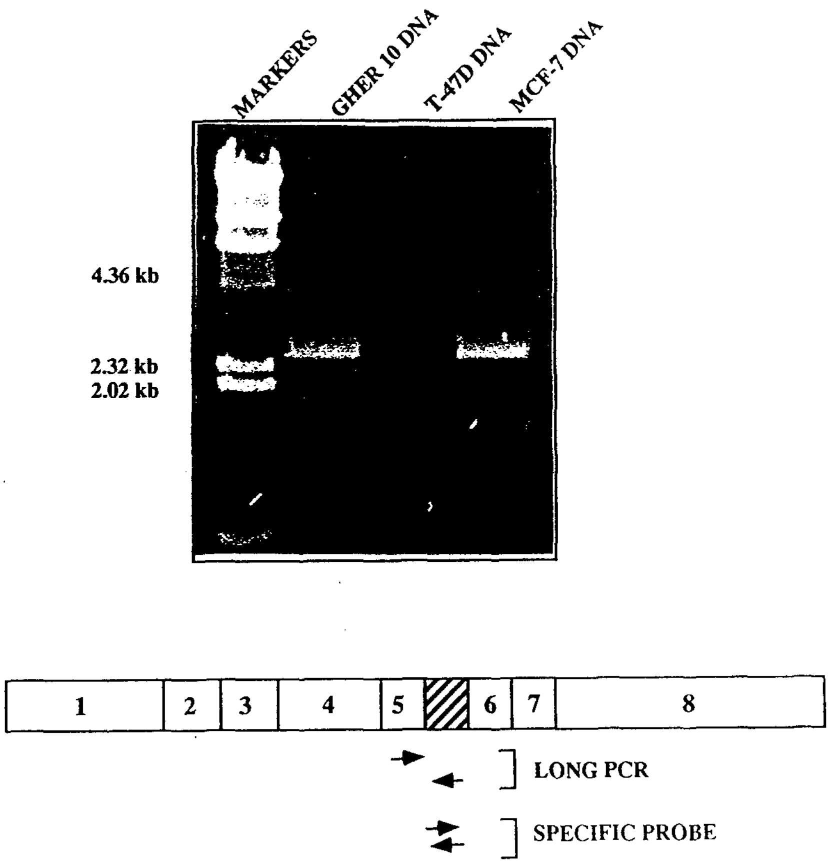


Figure 2

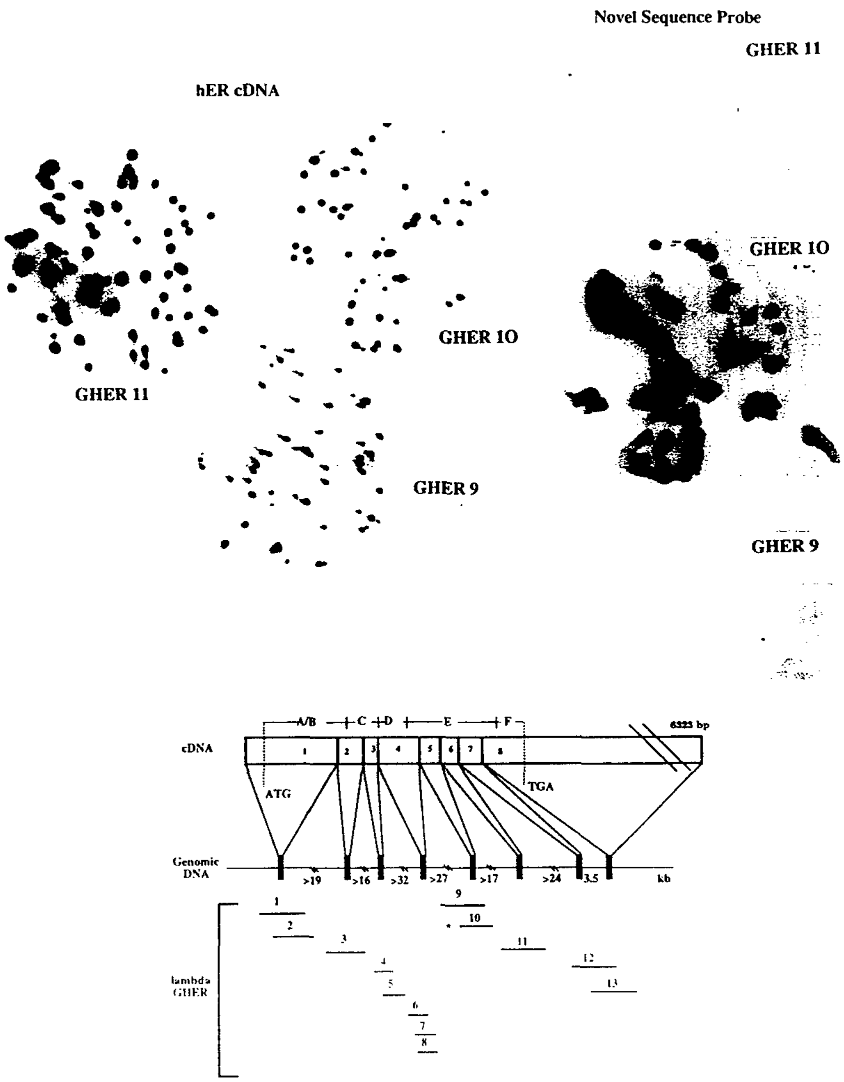

Novel Sequence Probe
GHER 11
hER cDNA
GHER 10
GHER 11
GHER 9
GHER 10
GHER 9
A/B
C
D
E
F
6323 bp
cDNA
ATG
TGA
Genomic DNA
>19
>16
>32
>27
>17
>24
3.5
kb
lambda GHER

FIGURE 3 Wang *et al.*,

EXON 5 GT...........................//.....................

1 TTGCC TAGAA AGATG TGACT TTGCT CCTTC TTCCC CTAGA TTGTG AGGCT TCCCC

56 AGTCA CAAGG AACTG TGAGT CCATT AAACC TTTTT CTTTG TAAAT TA**CCC AGTCT**

111 **CAGGT AGGTC TTT**AT TAGCA GCTTG AGAAC AGACT AATAC AAGGG GCTGT CTTGT

166 GCATT TT**AG***G ATGTT TAACA GCATC CCTGG ACTCC ACCAG CTAGC TGCCA GTAGC*

221 *AACCT CCACT TCCTC CAGTT ACG***AT** AACTA ACAAT GTCTC CGGAC ATTTC TACCT

276 ATCTT CTGTT GTGTC AGGGG GTGGG GGAGG TAAAA TTGCC TCTGG TTGAG AATCA

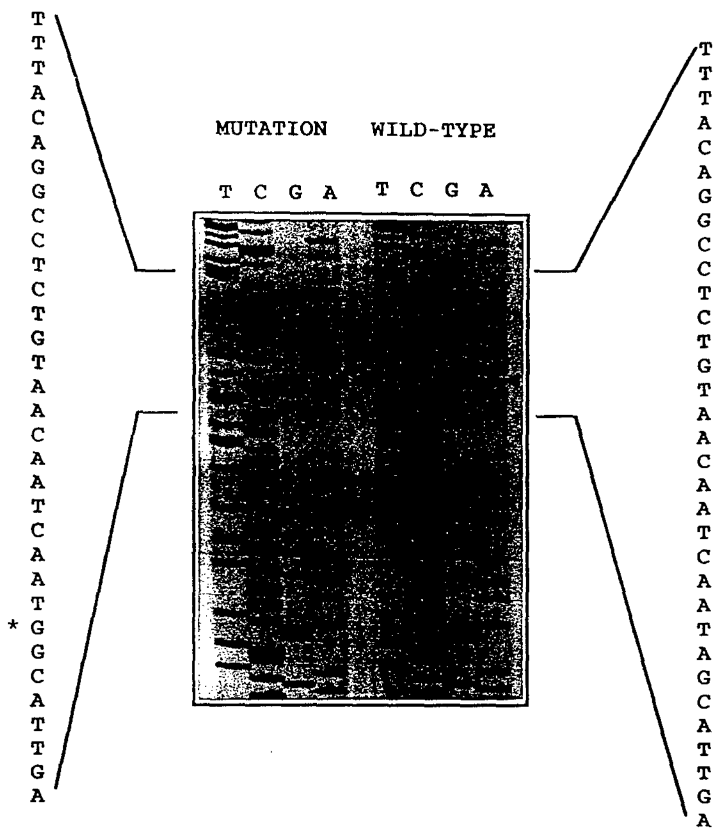
331 **CCACT CTATG CTTTC CCAAC TC**AAT GTTCT ATAAG CTCCT CAGAC ACACT GTACT

386 CTAAA CTGCA CACTC ACTTT ATCTT CTGCA ACAAG TCTAT TCCTT TTGGT TTTCT

441 GTCTT GGTGA............//........................AG EXON 6

Figure 4

A.



B.

AT

TAGCA GCTTG AGAAC AGACT AATAC AAGGG GCTGT CTTGT

GCATT TTAG*G ATGTT TAACA GCATC CCTGG ACTCC ACCAG*

*CTAGC TGCCA GTAGC AACCT CCACT TCCTC CAGTT ACG***G**T

AACTA ACAAT [illegible] CGGAC ATTTC TACCT ATCTT CT

APPENDIX 3

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Notes

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Estrogen Receptor Variants in Normal Human Mammary Tissue

*Etienne R. Leygue, Peter H. Watson, Leigh C. Murphy**

Background: Several estrogen receptor (ER) variant messenger RNAs (mRNAs) have been identified previously in human breast cancer biopsy samples and cell lines. The relative levels of certain ER variant mRNAs have been observed to increase with breast tumor progression. In vitro assays of the function of polypeptides encoded by some of these variant mRNAs have led to speculation that ER variants may be involved in the progression from hormone dependence to independence in breast cancer. _Purpose:_ We set out to establish if ER variant mRNAs are present in normal human breast tissues and, if so, to compare levels of these variants between normal and neoplastic human breast tissues. _Methods:_ Four human breast tissue samples from reduction mammoplasties and five samples from tissue adjacent to breast tumors were analyzed. The tissue samples were confirmed to be normal (i.e., not malignant) by histopathologic analysis. RNA was extracted immediately from adjacent frozen sections. Human breast tumor specimens originally resected from 19 patients were acquired from a tumor bank and processed in the same way as the normal tissue samples. The RNAs were then reverse transcribed and subsequently amplified with the use of the polymerase chain reaction (PCR). PCR primer sets were designed to detect several different exon-deleted ER variants and a truncated ER variant (i.e., clone 4). A semiquantitative PCR-based method was used to determine the relative expression of exon 5- and exon 7-deleted variants to wild-type ER mRNAs in the nine normal breast tissues and in 19 ER-positive breast tumor tissues. The Mann–Whitney rank sum test (two-sided) was used to determine _P_ values. _Results:_ ER variant mRNAs corresponding to the clone 4 ER truncated variant and to variants deleted in either exon 2, exon 3, exons 2-3, exon 5, or exon 7 were detected in all normal samples. The results were confirmed by restriction enzyme analyses and sequencing of the PCR products. The expression of exon 5-deleted ER variant relative to the wild-type ER mRNA was significantly lower ($P<.001$) in normal tissue than in tumor tissue. A similar trend was noted for expression of the exon 7-deleted ER variant mRNA; however, the difference did not achieve statistical significance ($P = .476$). _Conclusion:_ Several ER variant mRNAs are present in normal human breast tissue, but the level of expression of some of these variants may be lower in normal tissue than in tumor tissue. _Implication:_ These data suggest that the mechanisms generating ER variant mRNAs exist in normal breast tissue and may be deregulated in breast cancer tissues. Further investigation of the role of variant ER expression in development and progression of human breast cancer appears warranted. [J Natl Cancer Inst 1996; 88:284-90]

The estrogen receptor (ER), which belongs to the superfamily of steroid–thyroid–retinoic acid receptors (*1*), is an important regulator of growth and dif-

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See "Notes" section following "References."

ferentiation of the mammary gland. The receptor can be divided into several structural and functional domains (A-F) (*2*). In the absence of hormone, the receptor is thought to be associated with a protein complex, including heat-shock proteins such as hsp90 (*3*). On binding of ligand, the ER dissociates from this complex, dimerizes, and binds to specific sequences (estrogen receptor element or ERE) located in the 5′ flanking region of ER-responsive genes. Such interactions alter the transcription of estrogen-responsive genes. Region E of the receptor is implicated in ligand binding, dimerization, and *trans*-activating functions (TAF-2). The DNA-binding domain is located in the C region; another cell- and promoter-specific *trans*-activating function (TAF-1) lies within the A/B regions. Region D is involved in the nuclear localization of the receptor.

Several ER variant messenger RNAs (mRNAs) have been identified previously in human breast cancer biopsy samples and cell lines (*4-7*). While it is unclear if these mRNAs are translated in vivo, some of the predicted ER-like proteins, lacking some domains, exhibit altered functions or may interfere with wild-type (WT) ER function when recombinantly expressed. Exon 3- and exon 7-deleted variants were shown to act as dominant-negative regulators of WT ER (*8,9*). In contrast, exon 5-deleted ER has ligand independent *trans*-activating activity in a yeast-expression system (*10*). It has been shown that the relative levels of some of these ER variants were increased during tumor progression. Exon 5-deleted ER variant mRNA expression was found to be higher in ER-negative/progesterone receptor (PR)-positive tumors than in ER-positive/PR-positive tumors (*6,7*). Furthermore, exon 7-deleted variant mRNA levels were shown to be higher in ER-positive/PR-negative than in ER-positive/PR-positive tumors (*9*). Additional data from our laboratory suggest that elevated expression of another ER variant, called clone 4 truncated ER variant (*11*), is correlated with parameters of poor prognosis and endocrine insensitivity (*12*). It has thus been speculated that these ER variants may be involved in progression from hormone dependence to independence in breast cancer (*13*). However, the expression of ER variants in normal human breast tissue remains unknown. A recent study (*14*) shows the detection of several exon-deleted ER mRNAs in a single normal breast tissue sample. The investigators did not exclude the eventual development of breast cancer in the individual from whom the sample was derived. Moreover, no data were provided addressing relative expression of ER variants between normal and tumorous breast tissues. It was, therefore, important to establish definitively the existence of these ER variants in multiple normal breast tissue samples as well as to determine their relative level of expression between normal and tumorous breast tissues.

The aim of this study was to determine if multiple ER variant mRNAs can be detected in normal human breast tissue and, where possible, to compare the level of expression in normal tissues with that observed in tumor tissues.

Materials and Methods

Human Breast Tissues and Cell Lines

Normal breast tissues were obtained from reduction mammoplasty surgical specimens collected at the Necker Hospital, Paris, France (four case patients) and from normal tissues adjacent to tumors in mastectomy specimens obtained through the Manitoba Breast Tumor Bank, University of Manitoba, Winnipeg, Canada (five case patients). Human breast tumor specimens were also obtained from the Manitoba Breast Tumor Bank (19 case patients). In all case patients, the specimens had been rapidly frozen at −70 °C as soon as possible after surgical removal. Subsequently, a portion of the frozen tissue from each case patient (normal and tumor) was processed to create formalin-fixed and paraffin-embedded tissue blocks that were matched and orientated relative to the frozen tissue. These blocks provided tissue for high-quality histologic sections for pathologic interpretation and assessment. The presence of normal ducts and lobules was confirmed in all normal tissue specimens as well as the absence of any atypical lesion. The 19 primary invasive ductal carcinomas that were selected from the Manitoba Breast Tumor Bank database were all associated with high ER levels (105-284 fmol/mg protein). Within this group, 10 tumors were PR positive, four were PR negative, and five were borderline positive (<15 fmol/mg protein) as determined by ligand-binding assay. Specific frozen tissue blocks were chosen in each case on the basis of additional criteria as assessed in histologic sections. These criteria included the following: a cellular content of more than 30% invasive tumor cells with minimal normal lobular or ductal epithelial components, good histologic preservation, and absence of necrosis. In all tumors, grading was performed with the use of the Nottingham grading system (*15*), and additional clinical and staging information (e.g., patient age, tumor size, and nodal status) was obtained from the Tumor Bank database. The age distribution of patients associated with the normal samples was similar to that of the tumor group (mean, 70.2 years of age; standard deviation, 13 years). For reduction mammoplasties, women were younger (mean, 20 years of age; standard deviation, 3 years).

Ishikawa cells, an endometrial adenocarcinoma cell line (*16*) initially established by H. Iwasaki (Tsukuba, Japan), were provided by E. Gurpide (Mount Sinai School of Medicine, New York, NY). The breast cancer cell line T-47D-5 (*17*) was provided by R. L. Sutherland (Garvan Institute for Medical Research, Sydney, Australia). These cells are known to express different ER variant mRNAs (*17,18*; our unpublished data) and have therefore been used as positive controls. Cos-1 cells (American Type Culture Collection, Rockville, MD) do not express ER mRNAs and were used as a negative control in our experiments. Cells were grown and harvested, and cell pellets were stored at −70 °C as previously described (*19*).

Extraction of mRNA and Reverse Transcription

Total RNA was extracted from histologically defined regions within 20-μm frozen normal and tumor cryostat sections with the use of a small-scale RNA extraction protocol (Trireagent, MRCI, Cincinnati, OH) according to the manufacturer's instructions. The yield was quantitated by spectrophotometer in a 50-μL microcuvette. The average yield of total RNA per 20-μm section was 4 $\mu g/cm^2$ for tumor and 0.6 $\mu g/cm^2$ for normal tissues (± 20% variation with cellularity); the optical density ratios (260 nanometers/280 nanometers) of the RNA preparations were greater than 1.8.

Reverse transcription was performed in a final volume of 15 μL. RNA (600 ng) was reverse transcribed in the presence of 1 m*M* deoxyadenosine triphosphate (dATP), 1 m*M* deoxycytidine triphosphate (dCTP), 1 m*M* deoxyguanosine triphosphate (dGTP), 1 m*M* deoxythymidine triphosphate (dTTP), 5 m*M* dithiothreitol (Life Technologies, Inc., [GIBCO-BRL], Gaithersburg, MD), 1 U/μL ribonuclease inhibitor (Promega Corp., Madison, WI), 20 μ*M* random primers, 50 m*M* Tris–HCl (pH 7.5), 75 m*M* KCl, 3 m*M* $MgCl_2$, and 5 U/μL Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) for 10 minutes at 22 °C and 1 hour at 37 °C. After 5 minutes at 95 °C, 1 μL of the reaction mixture was taken for subsequent amplification with the use of the polymerase chain reaction (PCR).

Primers and PCR Conditions

Four sets of primers were used in this study (Fig. 1). The primer set that detected exon 5-deleted ER mRNA was called the D5 set and consisted of D5U primer (5′-CAGGGGTGAAGTGGGGTCTGCTG-3′; sense; located in WT ER exon 4; 1060-1082) and D5L primer (5′-ATGCGGAACCGAGATGATGT-AGC-3′; antisense; located in WT ER exon 6; 1520-1542). This primer set allows amplification of 483-base-pair (bp) and 344-bp fragments corresponding to WT ER and exon 5-deleted ER variant(s), respectively. The primer set designed to

detect exon 7-deleted ER mRNA was called the D7 primer set and consisted of D7U primer (5'-TCCTGATGATTGGTCTCGTCTGG-3'; sense; located in WT ER exon 5; 1389-1411) and D7L primer (5'-CAGGGATTATCTGAACCGTGTGG-3'; antisense; located in WT ER exon 8; 2035-2057). This primer set allows amplification of 668-bp, 534-bp, 484-bp, and 350-bp fragments corresponding to WT ER, exon 6-deleted, exon 7-deleted, and exon 6-7-deleted ER variants, respectively. The primer set that detects exon 2-, exon 3-, and exon 2-3-deleted ER mRNAs was called D2/3 primer set and consisted of D2/3U primer (5'-TGCCCTACTACCTGGAGAA-3'; sense; located in WT ER exon 1; 615-633) and D2/3L primer (5'-TGTTCTTCTTAGAGCGTTTGA-3'; antisense; located in WT ER exon 4; 1125-1145). This primer set allows amplification of 531-bp, 414-bp, 340-bp, and 222-bp fragments corresponding to WT ER, exon 3-deleted, exon 2-deleted, and exon 2-3-deleted ER variants, respectively. Primers designed to specifically detect clone 4 truncated ER mRNA consisted of clone 4 U primer (5'-TGCCCTACTACCTGGAGAA-3'; sense; located in WT ER exon 1; 623-641) and clone 4 L primer (5'-GGCTCTGTTCTGTTCCATT-3'; antisense; 941-959). This set allows amplification of a 337-bp fragment corresponding specifically to clone 4 truncated ER variant mRNA. Positions given correspond to published sequences of ER complementary DNA (cDNA) (*20*) and clone 4 cDNA (*11*).

PCR amplifications were performed with the use of 1 µL of reverse transcription mixture in a final volume of 50 µL, in the presence of 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.2 m*M* dATP, 0.2 m*M* dTTP, 0.2 m*M* dGTP, 0.2 m*M* dCTP, 4 ng/µL of each primer, and 1 U of *Taq* DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 40 cycles (1 minute at 60 °C, 30 seconds at 72 °C, and 30 seconds at 94 °C) on a Thermocycler (The Perkin-Elmer Corp., Foster City, CA). PCR products were then separated on 2% agarose gels before staining with ethidium bromide (15 µg/mL).

Identification of PCR Products

PCR products were identified by restriction enzyme analysis and sequencing. Bands corresponding to the clone 4 and exon 7-deleted ER variants were excised from gels after staining with ethidium bromide. The corresponding DNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) and precipitated with 70% ethanol in the presence of 100 m*M* CH_3COONa. PCR-amplified DNA product corresponding to clone 4 was digested for 18 hours at 37 °C with *Taq*I alone or *Taq*I plus *Kpn*I (5 U each per microgram of DNA). The PCR product corresponding to exon 7-deleted ER variant was digested under similar conditions with *Pst*I. Digestion products were separated on 2% agarose gels and their sizes were determined by reference to size markers (ΦX174 RF DNA/Hae III fragments; Life Technologies, Inc.). In parallel, approximately 50 ng of DNA was sequenced using clone 4 and D7 primer sets and dsDNA cycle sequencing system (Life Technologies, Inc.) according to the manufacturer's instructions.

For exon 5-, exon 2-, exon 3-, and exon 2-3-deleted ER variants, the PCR products were labeled with [α-^{32}P]dCTP (*see* below). One microliter of PCR product corresponding to an exon 5-deleted ER variant was digested with *Hin*dIII as described above. Similarly, PCR products corresponding to exon 2-, exon 3-, and exon 2-3-deleted ER variants were digested with *Taq*I alone or *Taq*I plus *Hha*I. Digestion products were separated on 6% polyacrylamide gels containing 7 *M* urea (PAGE). After electrophoresis, the gels were dried and exposed to Kodak XAR film at −70 °C with an intensifying screen, and the size of digestion products was determined by reference to size markers. In parallel, slices of gel corresponding to each labeled PCR product were excised from the dried gel and rehydrated overnight in 100 µL of sterile water. For each sample, five different PCR reactions were performed as described above with the use of 1 µL of this solution previously boiled for 10 minutes. PCR products corresponding to each set of five reactions were pooled, purified (Wizard PCR preps kit, Promega Corp.), cloned with the use of an Invitrogen TA Cloning kit, and sequenced as previously described (*11*).

Labeling of PCR Products

To label PCR products, a standard PCR reaction was performed in 10 µL supplemented with 10 n*M* [α-^{32}P]dCTP (ICN Pharmaceuticals, Inc., Irvine, CA). A 2-µL aliquot of the reaction was denaturated in 80% formamide buffer and subjected to PAGE. After electrophoresis, the gels were dried and autoradiographed for 6-18 hours.

Quantification and Statistical Analysis

The method used to quantitate exon-deleted variant mRNAs relative to WT ER mRNA is a modification of a method described by Daffada et al. (*21,22*). They showed that coamplification of WT ER and exon 5-deleted variant generates two DNA fragments whose ratio was constant with varying cycle numbers. This assay provides a semiquantitative reverse transcribed (RT)-PCR, whose internal control is the WT ER mRNA coamplified and in which relative expression of variant mRNA can be determined for individual samples. In our study, quantification of signals was carried out after excision of the band corresponding to variant and WT mRNA, followed by the addition of 5 mL scintillant (ICN Pharmaceuticals, Inc.), and counted in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The exon-deleted signal was expressed as a percentage of the WT ER signal. Preliminary studies showed that for each sample, the ratio of exon 5-deleted/WT ER signal and exon 7-deleted/WT ER signal remained constant (± 20%) and independent of the number of PCR cycles or initial cDNA input quantities. It should be noted that the percentage obtained reflects the relative ratio of variant to WT ER RT-PCR product and does not provide absolute initial mRNA levels.

For each sample, at least three independent assays were performed and the mean determined. The ratio of exon 5-deleted/WT ER signal and exon 7-deleted/WT ER signal measured during these assays never varied by more than 20%. The statistical significance of differences in the relative levels of expression of exon 5- and exon 7-deleted ER mRNAs in normal breast versus breast tumor tissue was determined with the use of the Mann–Whitney rank sum test (two-sided).

Results

Detection of ER Variants in Normal Breast Tissues

Total RNA from nine normal breast tissue specimens from nine different women was analyzed by reverse transcription-PCR with the use of the oligonucleotide primer pairs described in the "Materials and Methods" section and shown in Fig. 1. Primers were designed to allow the detection of different ER variants previously observed in breast cancer tissues or cell lines: exon 3-deleted (*8*), exon 5-deleted (*6*), and exon 7-deleted (*9*) ER variants and clone 4 ER truncated variant (*11*). These variants were detected with the use of D2/3, D5, D7, and clone 4 primer sets, respectively.

In the first series of experiments, PCR products were stained with ethidium bromide after separation on 2% agarose gel (Fig. 2). With the use of the D7 primer set (Fig. 2, A), two bands that corresponded in size to WT ER (668 bp) and to exon 7-deleted ER variant (Del 7, 484 bp) were obtained. These bands comigrated with those observed in the positive controls: T-47D-5 breast and Ishikawa uterine cancer cell lines. To confirm the identity of exon 7-deleted ER variant, the lower band was purified and digested with different restriction enzymes (data not shown). Nucleotide sequence obtained by cycle sequencing revealed a perfect boundary between exon 6 and exon 8 ER WT sequences (data not shown). These data definitively confirmed the identity of the exon 7-deleted ER PCR product obtained. With the use of the clone 4 primer set, a band migrating with the expected size of 337 bp was obtained (Fig. 2, B). Identity of this band was confirmed by enzymatic digestion and cycle sequencing (data not shown).

With the use of the D2/3 or D5 primer sets followed by ethidium bromide staining, no exon 3- and exon 5-deleted ER variant mRNAs were detected in normal tissues (data not shown). A more sensitive technique consisting of incorporation of labeled nucleotide into the PCR reaction followed by separation of PCR

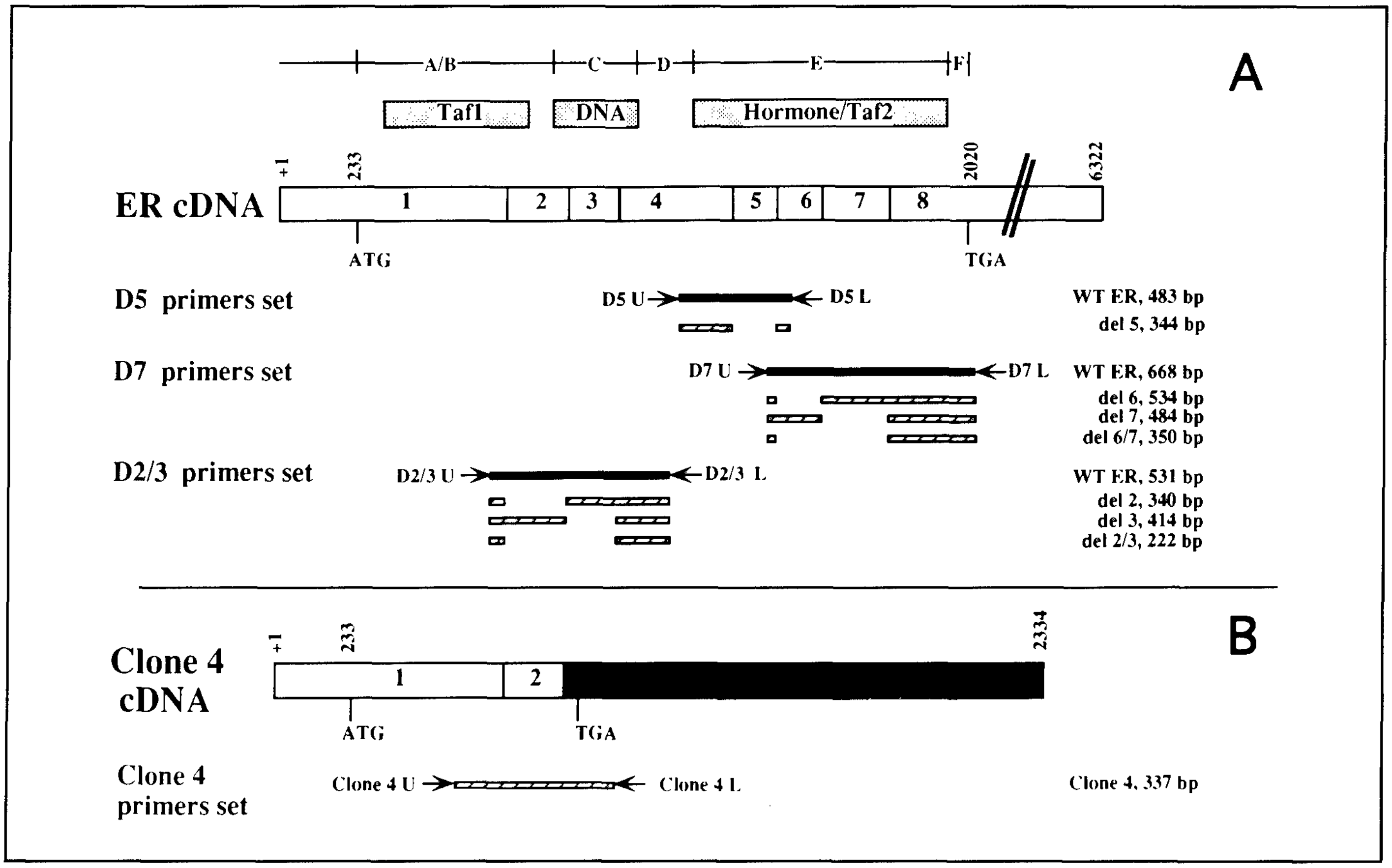

Fig. 1. A) Schematic representation of wild-type estrogen receptor (WT ER) complementary DNA (cDNA) and primers used to detect exon-deleted ER variants: ER cDNA contains eight different exons coding for a protein divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in *trans*-activating function (TAF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another *trans*-activating function (TAF-2). D5 primer set allows amplification of 483-base-pair (bp) and 344-bp fragments corresponding to WT ER and exon 5-deleted ER variants, respectively. D7 primer set allows amplification of 668-bp, 534-bp, 484-bp, and 350-bp fragments corresponding to WT ER, exon 6-deleted, exon 7-deleted, and exon 6-7-deleted ER variants, respectively. D2/3 primer set allows amplification of 531-bp, 414-bp, 340-bp, and 222-bp fragments corresponding to WT ER, exon 3-deleted, exon 2-deleted, and exon 2-3-deleted ER variants, respectively. **B**) Schematic representation of clone 4 ER variant cDNA and primers used to detect this variant: clone 4 cDNA contains the first two exons of ER cDNA followed by sequences with similarity to line-1 sequences (*17*). Clone 4 primer set allows amplification of 337-bp fragment corresponding specifically to clone 4-truncated ER variant mRNA.

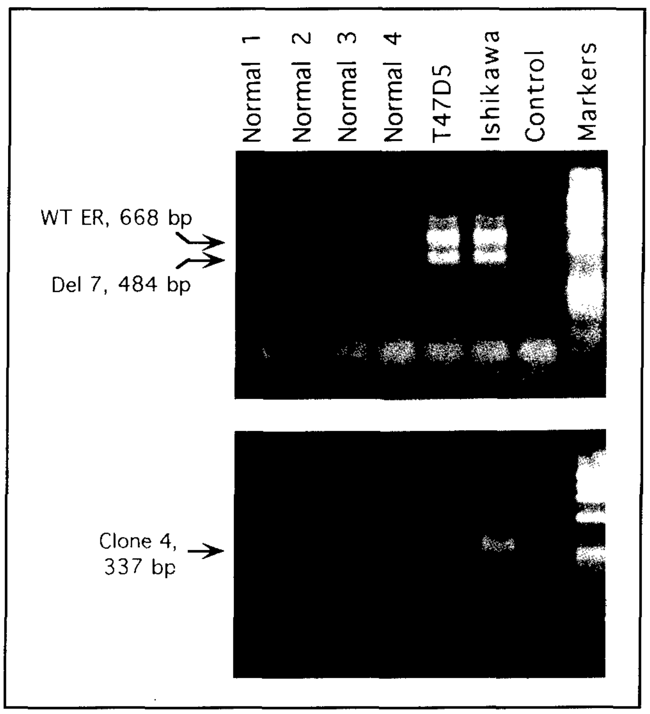

Fig. 2. Detection of exon 7-deleted and clone 4 ER variants in normal human breast: total RNA from normal human breast tissue samples (normal, 1-4), T-47D-5, and Ishikawa cancer cell lines, was reverse transcribed and polymerase chain reaction (PCR) amplified as described in the "Materials and Methods" section using the D7 primer set (**A**) and clone 4 primer set (**B**). PCR products were separated on 2% agarose gel before staining with ethidium bromide.

products on 6% denaturating polyacrylamide was subsequently used.

This technique, together with the D5 primer set, detected two bands corresponding in size to the WT ER mRNA (483 bp) and exon 5-deleted variant (344 bp) in all normal breast tissue samples (Fig. 3, A). Identity of the PCR products was confirmed following restriction enzyme digestion and sequencing (data not shown).

The D2/3 primer set and labeled PCR reactions resulted in the detection of four different PCR products in normal breast tissue samples. These products corresponded in size to WT ER (531 bp), exon 3-deleted (414 bp), exon 2-deleted (340 bp), and exon 2-3-deleted (222 bp) ER variant mRNAs (Fig. 3, C). Identity of these bands was confirmed with restriction enzyme digestion analysis and sequencing (data not shown).

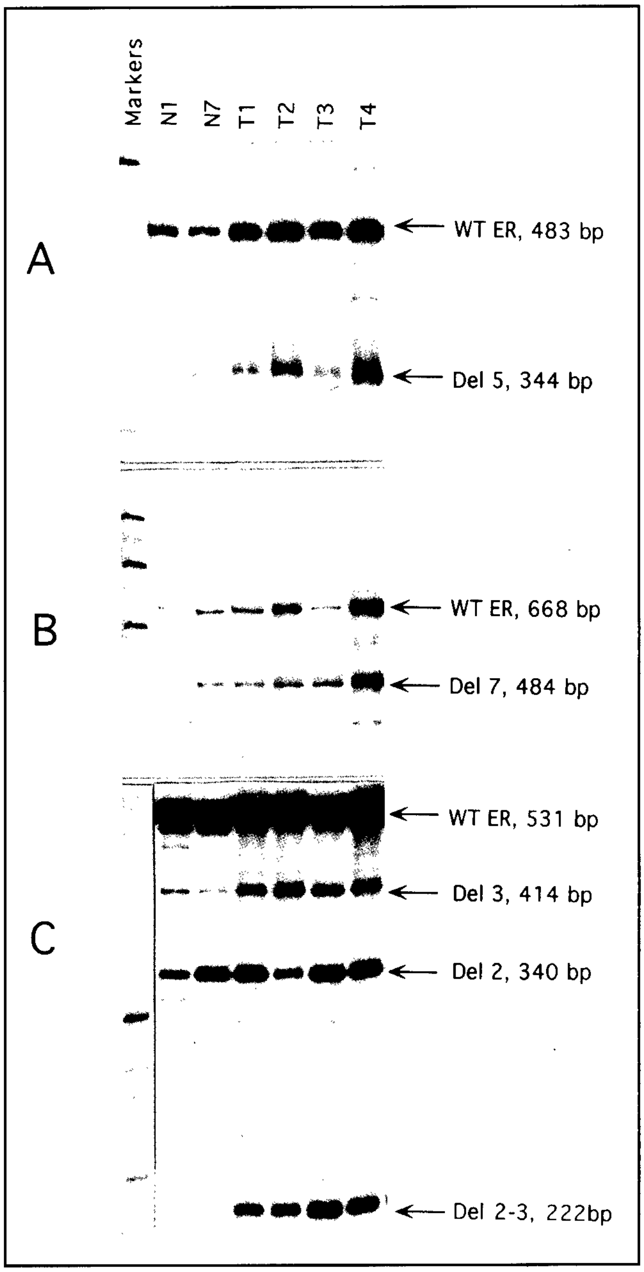

Fig. 3. Comparison of exon-deleted ER variants expression between normal and tumor breast tissues: total RNA from normal human breast tissue samples (N1-N2) and from breast tumors (T1-T4) was reverse transcribed and labeled polymerase chain reaction (PCR) amplification was performed as described in the "Materials and Methods" section with the use of the D5 primer set (**A**), D7 primer set (**B**), and D2/3 primer set (**C**). PCR products were separated on 6% polyacrylamide gel containing 7 *M* urea. After electrophoresis, the gel was dried and autoradiographed for 6-18 hours.

Comparison of Exon-Deleted ER Variant Expression in Normal and Tumor Tissues

The relative level of exon-deleted variant mRNA expression was compared in nine normal breast tissues and in 19 ER-positive breast cancer tissues. Expression relative to the WT ER mRNA was measured in each sample by incorporating a labeled nucleotide in the PCR products, which were then separated by PAGE (Fig. 3). Our preliminary studies confirmed the previous observation that amplification of WT and deleted variant transcripts occur with similar efficiency (*21,22*); therefore, the assay could be used to determine the relative levels of variant mRNA in individual samples. For exon 5- and exon 7-deleted variants, it was possible to express the signal measured as a percentage of the signal provided by the WT ER mRNA.

Because there was a substantial age difference between the patients who had reduction mammoplasty and the patients with breast cancer from whom normal breast tissue was taken, it was important to determine if the exon 5- and exon 7-deleted ER variant expression relative to WT ER in the normal breast tissues was identical irrespective of origin. No statistically significant difference was observed between these two subgroups of patients (data not shown).

The level of exon 5-deleted variant mRNA relative to the WT ER mRNA was found to be significantly less ($P<.001$) in normal (median, 21%) than in neoplastic breast cancer (median, 35%) tissues (Fig. 4).

Although a similar trend was observed for the exon 7-deleted variant between normal (median, 88%) and breast cancer (median, 107%) tissues, the difference failed to reach statistical significance ($P = .476$) (Fig. 4).

While expression of exon 2-, exon 3-, and exon 2-3-deleted variants was reproducibly observed in normal tissues, their relative expression changed from experiment to experiment, suggesting that the efficiency of reverse transcription-PCR varied when determination of relative expression of three different transcripts was attempted simultaneously. Fig. 3, C shows an experiment performed where the exon 2-3-deleted variant was not detected in normal sample N7. This could be explained by similarly low equivalent transcript levels of these variant mRNAs in normal tissues—the amplification occurring randomly on one variant rather than another. It was, therefore, not possible to quantify relative expression of these variants in normal tissues compared with that seen in tumor tissues.

Discussion

With the use of reverse transcription-PCR, it was possible to observe five different exon-deleted ER variant mRNAs and one truncated ER variant mRNA in each normal tissue studied. During the preparation of this manuscript, a paper describing the detection of exon 2-, exon 3-, and exon 7-deleted variant ER mRNAs in one normal human breast tissue sample was published (*14*). Our data confirm these observations and add new information concerning the expression of these variants in multiple normal human breast tissue samples. Furthermore, we have detected the expression of an exon 5-deleted ER transcript in multiple normal human tissue samples, and we have identified a previously unknown deletion variant (exon 2-3 deleted) in normal and breast cancer tissues. These two variants were not observed in the study by Pfeffer et al. (*14*). Our ability to detect these variants in normal tissues is probably due

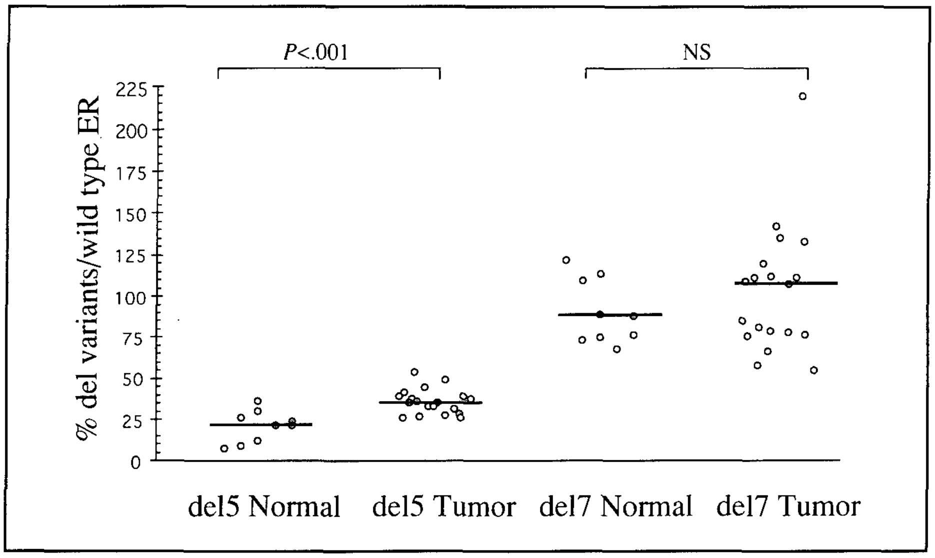

Fig. 4. Comparison of exon 5- (del5) and exon 7-deleted (del7) ER variant expression between normal and tumor breast tissues: total RNA from nine normal human breast tissue samples and from 19 human breast tumors was analyzed using the D5 primer set and D7 primer set as described in Fig. 3. Quantification of signals was carried out after excision of the band corresponding to variant and wild-type (WT) messenger RNA (mRNA), as described in the "Materials and Methods" section. The exon-deleted signal was expressed as a percentage of the WT ER signal. For each sample, at least three independent assays were performed and the mean was determined (circles). Differences in exon 5- and exon 7-deleted ER relative expression were then compared using the Mann–Whitney rank sum test (two-sided). Bar = median of each group. NS = nonsignificant.

to our use of a highly sensitive technique. The detection of ER variants in each of nine different normal tissue samples strongly suggests that the mechanisms generating these variants already exist in normal breast tissue. The primer sets we have used could potentially detect exon 6- and exons 6-7-deleted ER variants, but these were not observed. This suggests that the mechanisms used to generate deletion and truncated ER variants display some specificity and that generation of ER variants may have some role in normal ER regulation and/or function.

Many of these variants have been suggested to be involved in progression from estrogen dependence to independence in breast cancer (*7,23-25*). However, acquisition of hormone independence often occurs late in tumorigenesis; therefore, it was of interest to compare the expression of these ER variants between normal and cancer tissues with characteristics of good prognostic (i.e., ER positive) to gain further insight into their function and possible involvement in early tumorigenesis.

To have a representative group of ER-positive breast tumors, selection was made so that approximately one half of the speciments were ER positive/PR positive and the other half were ER positive/PR negative. While no significant difference in exon 5-deleted ER variant expression was observed between the two groups, the tumor group as a whole had significantly higher levels of exon 5-deleted variant expression relative to WT transcript than in normal breast tissues. Similarly, with the use of a new PCR-based quantitative method, we have recently demonstrated that clone 4 ER variant expression was increased in breast tumors compared with normal breast tissue (Leygue ER, Murphy LC, Watson PH: data submitted for publication). Taken together, these data suggest that the exon 5-deleted variant as well as clone 4 ER variant may have some role in early steps of tumorigenesis.

The absence of statistically significant differences between normal and breast tumor tissues with respect to the exon 7-deleted variant expression suggests that this variant may have a role in normal breast tissue that is not altered because of tumorigenesis. A similar finding, that altered expression of exon 5-deleted ER variant mRNA may occur in association with tumor progression while the same changes do not necessarily occur in the exon 7-deleted variant, has also been found in breast cancer cell lines that have developed tamoxifen resistance in vitro (*26*). This absence of difference may also be due to the small number of samples studied. Additional studies of larger numbers of normal breast and breast tumor tissue may be useful in clarifying this issue.

In conclusion, we have demonstrated that a range of ER variant mRNAs can be consistently detected in multiple independent samples of normal human breast tissues. Furthermore, by comparison between normal and neoplastic tissues, we have shown that the relative level of expression of specific variants in normal tissue can be altered and higher in tumor tissues. These data suggest that the mechanism(s) generating ER variant mRNAs already exist(s) in normal breast tissue and may be deregulated in breast cancer tissues; it is our speculation that such deregulation may contribute to progression in human breast cancer.

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Notes

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APPENDIX 4

[CANCER RESEARCH 56, 4324-4327, October 1, 1996]

Advances in Brief

Prevalence of Estrogen Receptor Variant Messenger RNAs in Human Breast Cancer[1]

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Abstract

A new approach, based on the competitive amplification of wild-type and exon-deleted estrogen receptor (ER) variant cDNAs, was used to screen 100 human breast tumors for the presence of ER variants. Already described exon 4-deleted ER mRNA was preferentially detected in tumors with lower grades ($P < 0.05$) or higher progesterone receptor levels ($P < 0.01$), whereas new ER variants, deleted in exons 2–4 or in regions within exons 3–7 were associated with higher grades ($P < 0.025$) and higher ERs ($P < 0.001$). This approach allows investigation of the expression of multiple ER variant mRNAs and may implicate them as new prognostic markers and as possible contributors to tumor progression.

Introduction

Several ER[3] variant mRNAs have now been detected in both normal and cancerous breast tissues (1–11). Although it is unclear if any or all of these mRNAs are translated *in vivo*, some of the predicted ER-like proteins, lacking some functional domains (12) of the WT-ER (Fig. 1), exhibit altered functions *in vitro*. Exon 3- and exon 7-deleted variants may act as dominant negative regulators of WT-ERs (3, 6), whereas exon 5-deleted ER has ligand-independent transcriptional activity (4, 13). Changes in the balance between ER-like molecules could be involved in perturbation of the ER signaling pathway and tumor progression (14–20). Many laboratories have begun to investigate the association between the expression of individual ER variant mRNAs and the loss of hormone-dependent growth (16, 19). However, it is now apparent that several different types of variant ER transcripts and therefore predicted proteins can be expressed together (8, 9), and the validity of investigating individual variants in isolation can be questioned. Furthermore, previous analyses have depended largely on assays that focus on limited regions of the transcript and that would be unlikely to detect more than one modification per individual variant mRNA. However, it is now clear that more than one modification can occur in variant transcripts (17). Thus, signals attributed to the exon 7-deleted ER variant mRNA detected with reverse transcription-PCR using primers in exons 5 and 8 (9) or with RNase protection assays with probes covering the exon 6–8 junction (20) may also include contributions from a variant deleted in both exons 4 and 7 recently identified by Madsen *et al.* (17). Nevertheless, these molecules may result in quite different proteins which differ in activity and modulate differentially the ER signaling pathway. Moreover, because of the lack of an approach to investigate qualitatively and quantitatively the representation of total ER variant mRNAs within any one given sample, it becomes difficult to evaluate those variants potentially important *in vivo* either as prognostic markers or as possible contributors to tumor progression. The purpose of this study was to develop a strategy that would allow the investigation of known and unknown exon-deleted or -inserted ER variant mRNAs in any one tissue sample as well as to determine possible changes in the relative expression of such variants among themselves and with respect to the WT-ER transcript. The approach used is depicted in Fig. 1. cDNAs corresponding to all exon-deleted ER variants identified to date can be amplified along with the WT-ER mRNA using primers annealing with exon 1 (1/8U) and exon 8 (1/8L) sequences. We assumed that a competitive amplification could therefore occur among all exon-deleted or -inserted ER variant transcripts that would depend on their initial relative representation, the detection of bands corresponding to specific ER variants reflecting the balance between ER variant mRNA species within the sample. Since it is likely that alterations in the coding sequences could be translated into ER-like proteins with altered functions, we have for practical reasons confined our approach to the coding region only. This approach was tested in this pilot study to determine the incidence of ER variants in a set of 100 breast tumors that were selected to represent a wide range of breast cancers with respect to ER and PR levels, size, grade, and axillary nodal status.

Materials and Methods

Human Breast Tissues and Cell Line. All human breast tumor specimens were obtained from the Manitoba Breast Tumor Bank. Tumors (100 cases) were chosen to represent a variety of tumor characteristics represented in the breast tumor population collected in the Manitoba Breast Tumor Bank. Thirty tumors were ER negative (ER < 3 fmol/mg protein), with PR values ranging from 0 to 25 fmol/mg protein, as measured using the ligand-binding assay. Seventy tumors were ER positive (ER ranging from 3.6 to 386 fmol/mg protein), with PR values ranging from 0 to 297 fmol/mg protein. These tumors also spanned a wide range of grades (from 4 to 9), determined using the Nottingham grading system (21), size (ranging from 1 to 6.3 cm), and nodal status (absence or presence of axillary nodes). T-47D-5 cells, which are known to express different ER variant mRNAs (11, 18), were kindly provided by Dr. R. L. Sutherland (Garvan Institute for Medical Research, Sydney, Australia). Total RNA was extracted and reverse transcribed in a final volume of 15 μl as described previously (11).

Primers and PCR Conditions. The primers used consisted of 1/8U primer (5′-TGCCCTACTACCTGGAGAACG-3′, sense; located in WT-ER exon 1; 615–637) and 1/8L primer (5′-GCCTCCCCCGTGATGTAA-3′, antisense; located in WT-ER exon 8; 1995–1978). Nucleotide positions given correspond to published sequences of ER cDNA (22). PCR amplifications were performed, and PCR products were analyzed as described previously (11), with minor modifications. Briefly, 1 μl of reverse transcriptase mixture was amplified in a final volume of 10 μl in the presence of 10 nM [α-^{32}P] dCTP, 4 ng/μl of each primer, and 1 unit

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[3] The abbreviations used are: ER, estrogen receptor; WT, wild type; PR, progesterone receptor; D2-,D3-, D4-, D5-, D7-ER, variant mRNA deleted in exons 2, 3, 4, 5, and 7, respectively; D3-4-, D2-3-ER, variant mRNA deleted in both exons 3 and 4 and in exons 2 and 3, respectively; D4/7-ER, variant mRNA deleted in both exons 4 and 7; D2-3/7-ER, variant mRNA deleted in exons 2, 3, and 7; D2-3-4-ER, variant mRNA deleted in exons 2, 3, and 4; D-3-7-ER, variant mRNA deleted in sequences within exon 3 to within exon 7; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

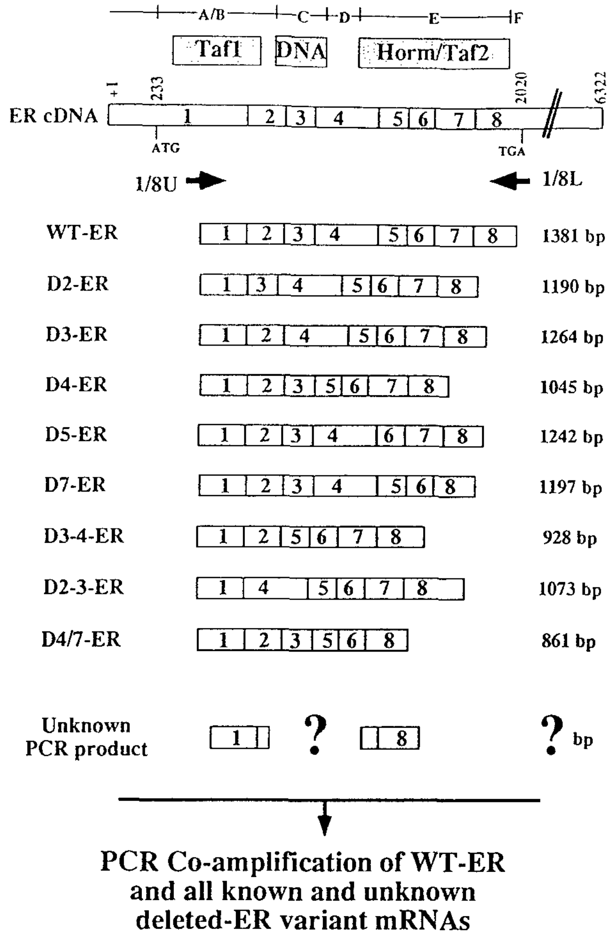

Fig. 1. Schematic representation of WT-ER cDNA and primers allowing coamplification of most of the described exon-deleted ER variants: ER cDNA contains eight different exons coding for a protein divided into structural and functional domains (*A–F*). *Region A/B* of the receptor is implicated in *trans*-activating function (*Taf1*). The DNA-binding domain is located in the *C region*. *Region E* is implicated in hormone binding and another *trans*-activating function (*Taf2*). 1/8U and 1/8L primers allow amplification of the 1381-bp fragment corresponding to WT-ER mRNA. Coamplification of all possible exon-deleted or -inserted variants which contain exon 1 and 8 sequences can occur. Amplification of the previously described ER variant mRNAs deleted in exon 2 (D2-ER), exon 3 (D3-ER), exon 4 (D4-ER), exon 5 (D5-ER), exon 7 (D7-ER), both exons 3 and 4 (D3-4-ER), exons 2 and 3 (D2-3-ER), exons 4 and 7 (D4/7-ER) would generate 1190-bp, 1264-bp, 1045-bp, 1242-bp, 1197-bp, 928-bp, 1073-bp, and 861-bp fragments, respectively.

of *Taq* DNA polymerase. Each PCR consisted of 40 cycles (1 min at 60°C, 2 min at 72°C, and 1 min at 94°C). PCR products were then separated on 3.5% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. To control for errors in the input of cDNA used in PCR reactions, amplification of the ubiquitous GAPDH cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide as described previously (11). All PCR products were subcloned and sequenced as described previously (11).

RNA Dilution Experiments. Plasmids that contained PCR products subsequently identified as fragments corresponding to exons 3- and 4-deleted ER variant (D3-4-ER) and to variant deleted in exons 2, 3, and 7 (D2-3/7-ER) were linearized with *Bam*HI and gel purified as described previously (11). Corresponding sense RNAs were synthesized using Riboprobe Systems (Promega, Madison, WI) according to the manufacturer's instructions. One μg of total RNA from T-47D-5 cells was mixed with various amounts of synthetic D2-3/7-ER (ranging from 5 ng to 50 fg) or D3-4-ER RNA (50 fg). These spiked RNA samples were then reverse transcribed and amplified using 1/8U and 1/8L primers as described above.

Statistical Analysis. Each individual tumor sample was analyzed in at least three independent assays. Only bands reproducibly observed in three experiments were considered. The presence of a specific band in a tumor sample was scored only if its signal intensity placed it among the four strongest signals (as assessed by subjective visualization) observed in the corresponding lane. The tumor group in which the band corresponding to the WT-ER mRNA was detected (68 cases) presented the following characteristics: ER level ranging from 0 to 386 fmol/mg protein (average, 111 fmol/mg protein) and PR level ranging from 0 to 297 fmol/mg protein (average, 73 fmol/mg protein). For the purpose of analysis, this group was divided into two subgroups presenting ER, PR, or a grade above or below a point defined as the average of the ER value, PR value, or grade observed within the group. Possible associations between the detection of a particular variant and one particular subgroup were tested using either the χ^2 test, including Yates' correction when the estimated frequency was at least equal to 5, or the Fisher exact test (two tailed) in other cases.

Results

Coamplification of WT-ER mRNA and Deleted Variant mRNAs in Breast Tumor Samples. On the basis of the assumption that coamplification of WT-ER mRNA and variant ER mRNAs could effectively occur and therefore allow identification of the frequency and relative expression of variants in breast tumor tissues, 100 breast tumors were selected for analysis that represented a wide range of ER and PR levels, as measured by the ligand-binding assay, grade, nodal status, and size. Total RNA was extracted from each tumor sample and reverse transcribed. PCR was then performed using primers annealing with exon 1 and exon 8 sequences. Fig. 2 shows typical results obtained. Many different PCR products were observed in each of 70 ER-positive tumors but only in 3 of 30 ER-negative tumors. This difference did not result from variable input of cDNA, since similar signals were obtained in all samples after amplification of the housekeeping GAPDH cDNA (data not shown). Two bands that migrated with the apparent sizes of 1381 and 1197 bp were observed in most of the signal-positive tumors. These bands were detectable in 68 and 63 cases, respectively. Following subcloning and sequencing, these bands were shown to correspond to the WT-ER and an exon 7-deleted ER (D7-ER) variant mRNA, respectively. Six other bands that migrated at the apparent sizes of 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp, and 580 bp were consistently detected within the set of tumors studied, but at an apparently lower frequency. They were observed in 19, 8, 6, 11, 6, and 20 tumors and were found to correspond to ER variant mRNAs deleted in exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3, and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2–4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. Sequences of all of these variants, except D-3-7-ER variant, showed a perfect junction between exons surrounding the deletion area (data not shown). A 801-bp deletion was observed in the D-3-7-ER variant from nucleotides 931 to 1729 (22) located within exon 3 and exon 7, respectively. It should be stressed that some bands, either not consistently observed or specific for less than three tumors, have not yet been assessed further in this study.

Detection of a Particular Variant Depends on Its Initial Representation within the ER mRNAs Population. To determine whether the detection of a variant depended on its initial representation within the ER-like mRNA population, the balance of ER-deleted variants was artificially changed in favor of particular variants. Various amounts of synthetic RNAs corresponding to the D3-4-ER and D2-3/7-ER PCR products were added to total RNA extracted from T-47D-5 breast cancer cells. These RNA preparations were reverse transcribed and subsequently analyzed with PCR using 1/8U and 1/8L primers (Fig. 3). Bands corresponding to WT-ER, D7-ER, D4-ER, and D-3-7-ER were initially detected in T-47D-5. The addition of synthetic D2-3/7-ER RNA, which increased its ability to compete for the binding of 1/8U and 1/8L primers during the PCR reaction, drastically decreased signals corresponding to the initially detectable endogenous variants. The extinction of these signals was directly related to the concentration of the synthetic RNA added. The Addition

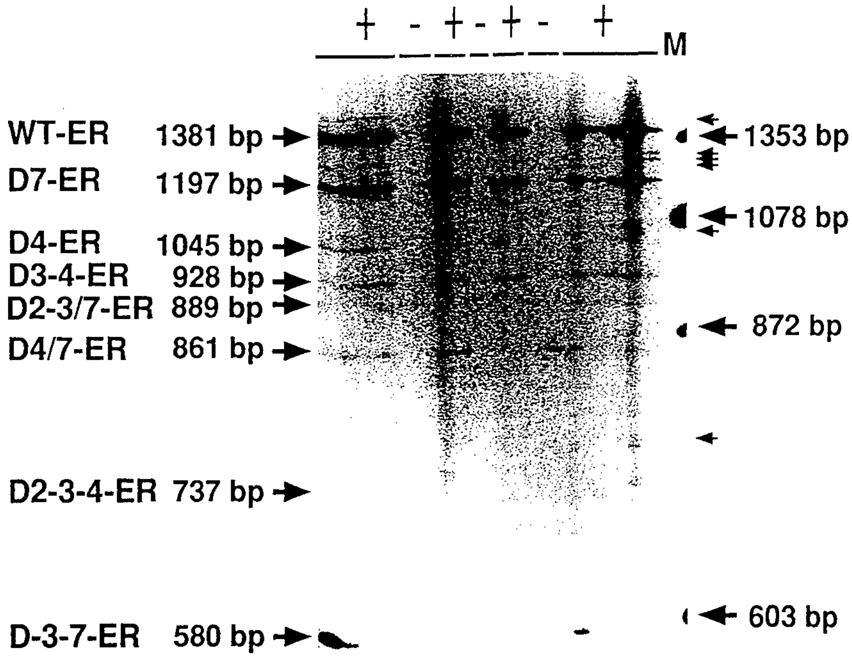

Fig. 2. Coamplification of WT-ER and deleted variant mRNAs in breast tumor samples: total RNA extracted from different ER-positive (+) and ER-negative (−) breast tumors was reverse transcribed and PCR amplified as described in "Materials and Methods" using 1/8U and 1/8L primers. Radioactive PCR products were separated on a 3.5% acrylamide gel and visualized using autoradiography. To each lane corresponds a unique tumor. Bands reproducibly obtained within the set of tumors studied and that migrated at 1381 bp, 1197 bp, 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp, and 580 bp were identified as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3, and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2–4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. PCR products indicated by *small arrows*, barely detectable within the tumor population, *i.e.*, present in less than or equal to three particular tumors, have not yet been identified. *M*, molecular weight marker (ϕX174; Life Technologies, Inc., Grand Island, NY).

of two synthetic RNAs simultaneously resulted in the increased representation of two expected bands.

Detection of Particular Variants May Be Associated with Tumor Characteristics. Detection of ER variants using the approach described here appeared to depend on the initial relative ratio of expression between ER-like mRNAs. It was therefore of interest to search for possible associations between the detection of particular variants and other tumor characteristics. The detection of a specific band in a sample was defined here as its presence as one of the four main signals observed in the corresponding lane. The frequency of detection of each ER variant mRNA within tumors also expressing a detectable WT-ER band is presented Table 1. Using the mean ER, PR, and grade values as cutoff points for statistical analysis, we found that D-3-7-ER and D2-3-4-ER variants were preferentially detected in the subgroup with higher ER ($P < 0.001$) and higher grade ($P < 0.025$), respectively. D4-ER variant was more frequently observed in tumors of lower grade ($P < 0.05$) or with higher PR levels ($P < 0.01$).

Fig. 3. Coamplification of WT-ER and deleted variant mRNAs after artificial modification of the balance between ER-like mRNAs: one μg of total RNA from T-47D-5 cells alone (*Lane 1*) or mixed with various amounts of synthetic D2-3/7-ER (5 ng, 500 pg, 50 pg, 5 pg, 500 fg, and 50 fg: *Lanes 4, 5, 6, 7, 8,* and *9*, respectively) and D3-4-ER (50 fg, *Lane 9*) RNAs. These spiked RNAs were then reverse transcribed and amplified as described in "Material and Methods." PCR products were separated on 3.5% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gel was dried and autoradiographed for 18 h. D2-3/7-ER and D3-4-ER RNAs alone (5 ng) were similarly analyzed (*Lanes 2* and *3*, respectively). *M*, molecular weight marker.

Discussion

We have used a new approach based on the competitive coamplification of WT-ER and exon-deleted or -inserted ER variant mRNAs to examine the overall expression of these two types of ER variants which encompass the majority of ER variant mRNAs thus far identified (23). Although another distinct group of variants, the truncated ER variants that include the widely expressed ER clone 4 variant (5, 18), cannot be assessed with this analysis, the strategy allows a broad investigation of the ER-like population and the integrity of the entire coding region within this species, without focusing on particular regions. This has enabled us to confirm the existence of four variants already described by others, *e.g.*, exon 7-deleted ER variant (3, 16), exon 4-deleted ER variant (7), exon 3–4-deleted ER variant (9), and a variant deleted in both exons 4 and 7 (17). Beyond these, three new variants were identified. Two of them, deleted in exons 2, 3, and 7 or exons 2–4, correspond to the usual exon-deleted ER variant pattern, *i.e.*, containing a perfect deletion of exon sequences. The third one contained part of exon 3 attached to a sequence beginning inside the seventh exon. It should be noted that very recently, Daffada and Dowsett (24) identified an ER variant presenting a similar pattern of intra-exon deletion between exons 4 and 7. Furthermore, we have been able to detect ER variant mRNA deleted in both exons 4 and 7 for the first time in multiple clinical material, supporting the potential relevance of such a variant *in vivo*. The function of the putative encoded protein which lacks a nuclear localization signal, all of the hinge domain, and is C-terminal truncated remains to be determined.

Using different RNA preparations, we showed that the detection of

Table 1 *Frequency of detection of ER variant mRNAs within 68 human breast tumors also expressing detectable WT-ER mRNA*

	Tumors expressing WT-ER	No. of tumors expressing detectable ER variant mRNAs						
		D7-ER	D4-ER	D3–4-ER	D2–3/7-ER	D4/7-ER	D2–3–4-ER	D–3–7-ER
ER < 111 (fmol/mg protein)	38	34	8	6	4	7	3	3
ER > 111 (fmol/mg protein)	30	27	9	2	2	3	2	16 $P < 0.001^a$
PR < 73 (fmol/mg protein)	41	35	5	7	5	6	4	10
PR > 73 (fmol/mg protein)	27	26	12 $P < 0.01^a$	1	1	4	1	9
$4 \leq$ grade ≥ 6	35	33	13 $P < 0.05^a$	4	3	5	0	10
$7 \leq$ grade ≥ 9	33	28	4	4	3	5	5 $P < 0.025^b$	9

WT and variant ER mRNAs were detected after co-amplification as described in "Materials and Methods."
[a] P values calculated using the χ^2 test with Yates' correction.
[b] P value calculated using the Fisher exact test (two tailed).

a variant depended on its initial representation within the ER-like mRNA population. The absence of a prominent signal corresponding to any particular variant could therefore result from its low relative representation. This could explain why variants deleted in either exon 3 or exon 5 were undetectable using our criteria and this approach, although their presence was confirmed by specific PCR amplification in some of the same tumors studied.[4] These variants may also correspond to infrequent or poorly represented ER-like mRNAs and therefore PCR products that we have not yet identified. On the other hand, the detection of any particular ER variant mRNA within a tumor sample can result from its overexpression or a change in the balance between all ER variant mRNAs. Using this approach, it is therefore possible to investigate the relative proportion of ER variant mRNAs, and also to compare breast samples regarding the relative expression of their ER-like mRNAs.

The set of tumors analyzed in this pilot study was chosen to obtain the widest qualitative representation of important breast tumor characteristics more than to establish statistical associations. The tumor population contained very different tumors spread over a wide range of ER and PR levels, size, grade, and nodal status. It was possible however to establish that detection of particular variants may be correlated with already known prognostic markers. It is interesting to note that the exon 4-deleted variant is associated in this study group with two different markers of good prognosis, *i.e.*, high PR and lower grade. This variant, initially described in breast cancer cell lines (7) and subsequently *in vivo* in several normal and tumor tissues (9, 10), is expected to encode an ER-like protein lacking most of the hinge domain, which includes an important nuclear localization signal and a part of the hormone binding domain. It might therefore have a cellular distribution and estrogen-binding affinity different from that of the WT-ER. Furthermore, the altered structure of this protein may lead to altered transcriptional activities.

The use of this approach to study a larger set of samples would allow the establishment of a typical pattern of ER variant mRNA expression for each type of tumor. Comparison of such patterns along with the subsequent analysis of the specifically detected transcripts could lead to the discovery of new prognostic factors and the identification of new contributors to tumor progression.

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[4] E. Leygue, unpublished data.

APPENDIX 5

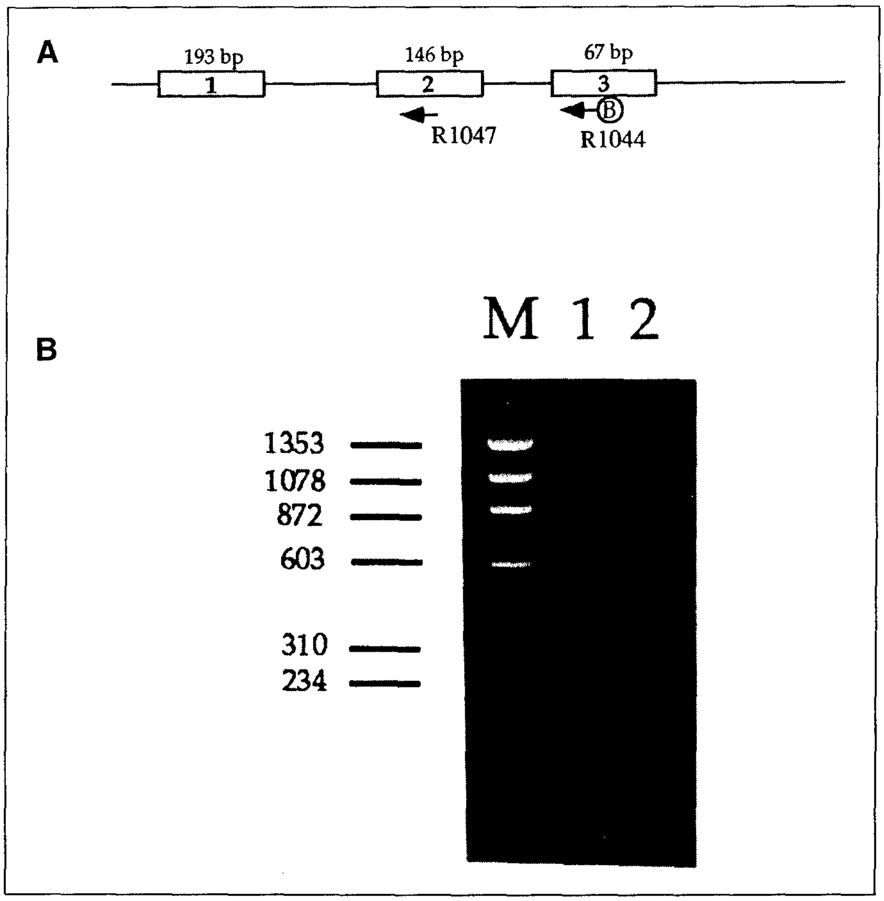

Figure 2. Isolation of 5′ end of the transcript from a human ubiquitin carboxyl-terminal hydrolase gene. (A) Schematic representation of the 5′ end of a human ubiquitin carboxyl-terminal hydrolase gene. (B) Human WBC mRNA was used in the LA-PCR. Amplification of anchored first-strand cDNA using the anchor-specific primer (T3) and R1044, which has a biotin at its 5′ end (lane 1), followed by a nested PCR using T3/R1047 primers (lane 2). Lane M contains the ϕX174 RF DNA *Hae*III fragments.

quenced, as indicated in Figure 2. In this case, the outer primer specific for the cDNA was synthesized with biotin at the 5′ end. The product of the first PCR was passed through QIAquick PCR purification columns to remove excess primers. The biotinylated PCR product was then captured by streptavidin-coated Dynabeads® M280 (Dynal, Oslo, Norway). An aliquot of the bead-captured DNA was used for the nested PCR (Figure 2).

In summary, the modifications of the LA-PCR described here make the procedure more reproducible and simplify the process by eliminating two enzymatic steps in the preparation of the anchor.

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Microdissection RT-PCR Analysis of Gene Expression in Pathologically Defined Frozen Tissue Sections

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Molecular studies of mRNA gene expression in human solid tumors are critically dependent on the ability to apply sensitive assays to tumor tissue that is of the highest quality with respect to pathological definition and cellular preservation. In particular, the interpretation of any analysis must recognize the problems that are posed by variability in cellular composition.

Although the level of mRNA can be assessed by a variety of techniques, many such as Northern blot and RNase protection assay are not sensitive enough to allow the study of small tumor samples that are usually available for research. Alternatively, in situ hybridization allows the assessment of individual cell expression. However, sensitivity, accurate quantitation and determination of mRNA structure can sometimes be a significant limitation. The reverse-transcription polymerase chain reaction (RT-PCR) assay offers a sensitive alternative that can allow accurate measurement of both structure and level of mRNA based on very small samples.

We and others have previously demonstrated the feasibility of extracting DNA from microdissected regions within archival formalin-fixed and paraffin-embedded tissue sections to assess alterations in gene structure by PCR (4,10,11). Several groups have

also reported RNA extraction from paraffin sections (1,6–9). However, this can require specialized approaches to tissue fixation (6), and our experience is that this allows the amplification of only relatively stable and abundant RNA species, such as "housekeeping genes" (3,8). Alternatively, RNA can be extracted from frozen tissues; however, this has the limitation of suboptimal histological detail for the assessment of tumor parameters and precise cellular composition. In this report, we describe an approach to facilitate microdissection of small pathologically defined regions from frozen tumor sections to provide mRNA for the analysis of gene expression by RT-PCR.

Fresh tissue samples from breast cancer cases are obtained through a standardized and timed collection protocol instituted by the National Cancer Institute of Canada - Manitoba Breast Tumor Bank. Portions of these tissues (typically 0.5 cm^3) are then rapidly bisected, orientated on the external and cut surfaces with different colored dyes (black india ink, alcian blue and mercurochrome) and one-half placed in 10% neutral-buffered formalin, and the other is snap-frozen in liquid nitrogen within a cryovial. The fixed-tissue blocks are then processed routinely to produce matching and "mirror image" formalin-fixed, paraffin-embedded and frozen tissue blocks. Thin (5 µm) hematoxylin and eosin (H&E)-stained sections are then prepared from the paraffin blocks to allow interpretation of the detailed histology and tumor composition by light microscopy. The corresponding frozen blocks can then be sectioned in a cryostat to provide thin 5- to 20-µm sections when required, in which the distinction of tumor grade, mitotic rate, in situ tumor vs. florid ductal hyperplasia and other subtle features can be determined by direct comparison with the adjacent high-quality paraffin section.

High-quality total RNA is extractable from whole 20-µm frozen tumor sections using a small scale extraction protocol (TRI Reagent™; Molecular Research Center, Cincinnati, OH, USA) to provide an average yield of 4 µg/cm^2/20-µm tumor section (consistently optical density $[OD]_{260/280}$ >1.8 as quantitated by spectrophotometer in a 50-µL microcuvette). Although this varies with the tumor cellularity, a typical tumor section measuring 0.25 cm^2 yields 1 µg of total RNA, which is sufficient to be used as a substrate for multiple RT-PCR assays. We have used this approach to reliably amplify a range of gene products such as *c-myc*, *pS2* and *CD*44. We have also examined the effect of reuse and storage on the yield and quality of RNA that can be extracted from frozen tissue sections. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and estrogen receptor (ER) gene expression were assessed by RT-PCR using RNA extracted from frozen tissue sections, obtained from four different tumor cases. These had previously been sectioned for successful RT-PCR analysis and then re-frozen from 1½ to 2½ years previously. We have found no significant loss of RT-PCR signal in RNA extracted from previously sectioned frozen blocks by comparison with the original RNA extracted (Figure 1) when the tissue blocks are stored at -70°C and carefully handled during processing. By comparison, and as illustrated by the tumor in Figure 1, lane 6, degradation of

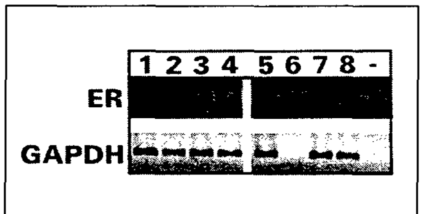

Figure 1. RT-PCR analysis of ER and GAPDH expression within frozen sections from resected tumor blocks. RNA was extracted from frozen sections obtained from tumor blocks that had been sectioned and successfully used for RNA extraction and RT-PCR up to 2½ years previously. In all four tumors, the expression of ER and GAPDH can be readily detected in the reused blocks (lanes 1–4), and this is similar to the expression seen in the corresponding samples of RNA stored at -70°C since the original extraction (lanes 5–8). Note that the integrity of one stored RNA sample (lane 6) has been lost.

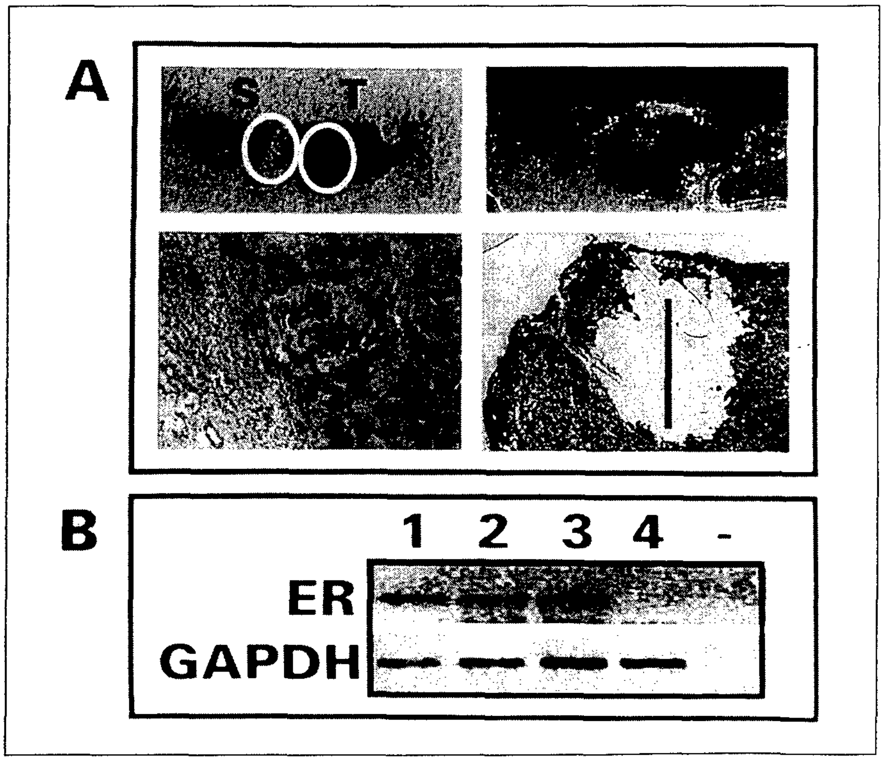

Figure 2. Microdissection/RT-PCR analysis of ER and GAPDH expression within a breast tumor section. Upper panel (A) shows original H&E-stained frozen section from a breast tumor with separate regions consisting of invasive tumor and stroma (top left); the adjacent serial section mounted on agarose after microdissection (top right); the detail of the histology of the lymphocyte-rich stroma and tumor (bottom left) and the microdissected tumor region (bottom right, scale bar = 0.2 cm). Lower panel (B) shows the results of RT-PCR analysis of ER and GAPDH expression from the entire frozen tumor section (lane 1), the entire H&E-stained frozen tumor section (lane 2), the microdissected region of invasive tumor (lane 3), the microdissected region of stroma (lane 4) and the RT-PCR negative control (RNA-).

stored RNA samples can occur through incomplete removal of endogenous tissue RNases or contamination by exogenous RNases introduced during RNA extraction or tube storage (5).

To assess gene expression in a defined region within a tissue section, several 20-μm frozen sections may be cut from the frozen tumor block (in a Leica cryostat at -30°C; Leica) and each then mounted onto a glass slide that has been previously coated with 2% agarose (Boehringer Mannheim, Laval, PQ, Canada) for microdissection using a modification of a method previously described for assessment of enzyme activity (2). Frozen sections directly mounted onto glass slides dry out rapidly during dissection and are difficult to dissect. Slides coated by 2% agarose, to a depth of 1 mm, can be prepared by pouring molten agarose in autoclaved ddH_2O onto slides. These are then stored at 4°C for up to 1 h before use to prevent dessication. Mounted sections are immersed in a Harris's hematoxylin solution (Mallinckrodt, Winnipeg, MB, Canada) for 2 s at room temperature, rinsed in water for 10 s, immersed into Eosin Y in 95% ethanol (Mallinckrodt) for 2 s, rinsed again in water for 10 s and then placed under a dissection microscope (wild M3C; Leica). Sections can then be oriented and the histological details confirmed with reference to a paraffin H&E-stained section from the matching paraffin block. Using a scalpel blade and fine needle, specific tumor components within histologically defined areas less than 1–2 mm^2 can be rapidly microdissected within 2 min under a 20× objective at room temperature and placed into a precooled microcentrifuge tube on ice. After a brief centrifugation to pellet the dissected material, 10 μL ddH_2O are added, and the material is snap-frozen by immersion in liquid nitrogen to disrupt tissue architecture. RNA can then be extracted and used as a substrate for RT-PCR assay. For a typical 20-μm breast tumor section, which may contain 10^4 cells within a 2-mm region, we obtain yields of between 0.5–1 μg RNA from 4 microdissected serial sections. The brief H&E stain provides essential cellular discrimination without significantly affecting the ability to perform successful RT-PCR.

To demonstrate the feasibility of this approach, a microdissection experiment performed on tissue from an ER-positive carcinoma is shown in Figure 2. The mRNA expression of the GAPDH "housekeeping" gene is detected in the entire unstained frozen tumor section, in the section following brief H&E staining and in both microdissected tumor and stroma regions. Similarly, expression of ER is seen in the entire tumor section and in the region of invasive carcinoma, but as expected, it is absent in the immediately adjacent lymphocyte-rich stroma.

Following microdissection, 100 ng total RNA from each component were reverse transcribed in a volume of 20 μL of RT mixture (1× RT buffer and 200 U Moloney murine leukemia virus [MMLV] RTase (Life Technologies, Burlington, ON, Canada); 0.5 mM each dGTP, dATP, dTTP, dCTP; 1 μM bovine serum albumin [BSA]; 0.01 M dithiothreitol [DTT]; 1.25 mM oligo(dT) primer; 5% dimethyl sulfoxide [DMSO]) and incubated for 60 min at 37°C. PCR amplification of GAPDH and ER cDNA was then performed in a Model PTC-100™ thermal cycler (MJ Research, Watertown, MA, USA). Each PCR was performed in a 50-μL volume utilizing 2 μL of the completed RT reaction containing cDNA; 1× PCR buffer; 2 mM $MgCl_2$; 1.1 U *Taq* DNA Polymerase (Promega, Unionville, ON, Canada); 200 mM each dGTP, dATP, dTTP and dCTP; and 0.5 mM PCR primers. The PCR protocol consisted of 5 min at 94°C; then 40 cycles of 45 s at 93°C, 45 s at 56°C and 90 s at 75°C; followed by 7 min at 72°C. After thermal cycling was completed, 1.5 μL of gel loading buffer were added to 15 μL of the PCR, and samples were electrophoresed on a 2% agarose gel. PCR products were visualized by subsequent ethidium bromide staining and photography under UV light. The primer sequences used were as follows; $GAPDH^{943}$ 5′ ACCCACTCCTCCACCTTTG 3′, $GAPDH^{1102}$ 5′ CTCTTGTGCTCTTGCTGGG 3′, ER^{675} 5′ TGCCCTACTACCTGGAGAA 3′, ER^{860} 5′ TGGTAGCCTGAAGCATAGTC 3′.

In conclusion, we have described an approach involving a specific protocol for tissue processing that allows for the extraction of mRNA from single histologically defined tumor sections. We have also shown that it is feasible to microdissect small areas from within H&E-stained frozen tumor sections and to extract RNA that is suitable for RT-PCR analysis of specific components within tumors.

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Dimethyl Sulfoxide Improves RNA Amplification

BioTechniques 21:44-47 (July 1996)

Reverse transcription and polymerase chain reaction (RT-PCR) are increasingly used for the detection and quantitation of various RNAs. We have used this method for optimizing the sensitivity of detection of a number of RNA viruses. During our work on amplification of RNA with the r*Tth* DNA Polymerase system from Perkin-Elmer (Norwalk, CT, USA), we have found that the presence of dimethyl sulfoxide (DMSO) in the cDNA formation step of the procedure greatly enhances the detection sensitivity and increases the yield of the amplified products. To our knowledge, this is the first report to demonstrate the effect of DMSO on r*Tth* DNA Polymerase-mediated RT-PCR. DMSO at less than 10% concentration has previously been demonstrated to be necessary in some cases (9) and enhances other Klenow- and *Taq* polymerase-mediated amplifications (10).

RNA templates to be used in RT-PCR were synthesized in vitro by using SP6 or T7 RNA Polymerase (Promega, Madison,WI, USA) from plasmid constructs containing part of the genomes of the viruses under study. HIV-1 RNA was prepared by synthesis of a full-length *gag* polyprotein cloned in an in vitro transcription/translation vector pDAB72 (1). This DNA was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD, USA). Human immunodeficiency virus type 2 (HIV-2) RNA was prepared from a molecular clone, PSXB1(pJSP4-27/H6) obtained from Dr. George Shaw

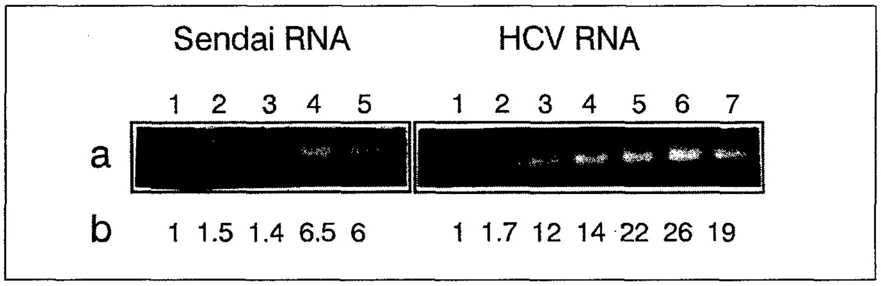

Figure 1. Effect of DMSO concentration on RT-PCR amplification of Sendai and HCV RNA. For Sendai RNA, lanes 1–5 contain 0, 2, 4, 6 and 8% DMSO, respectively, in the RT step. For HCV RNA, lanes 1–7 contain 0, 2, 4, 6, 8, 10 and 12% DMSO, respectively, in the RT step. a) Ethidium bromide-stained gel showing amplification of RNA. b) Fold increase in the density of each band is presented under each lane. Density was measured by scanning the negative lane of the ethidium bromide-stained gel. RNA template was added in the master mixture of each viral species and aliquoted to contain 10^6 copies of Sendai virus RNA per reaction and 5×10^6 copies of HCV RNA per reaction.

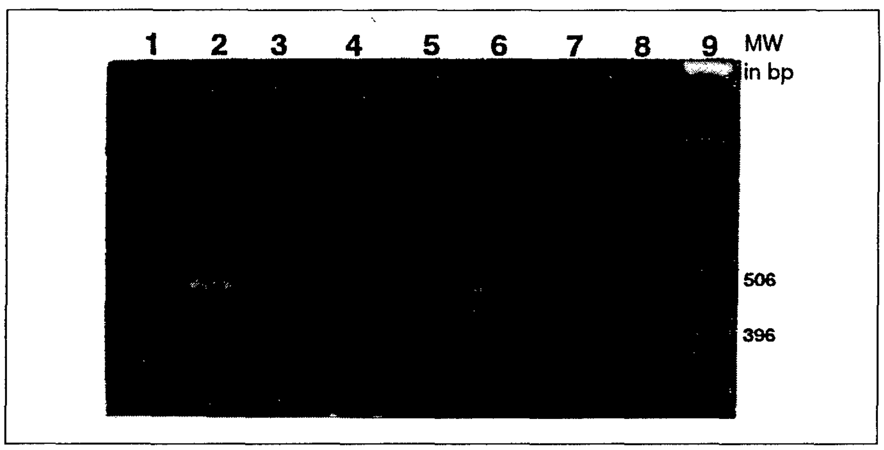

Figure 2. Effect of DMSO on RT-PCR amplification of HAV RNA. Lanes 1 and 3, without DMSO; lane 2, 5% in the RT step; lane 4, 1%; lane 5, 2%; lane 6, 5%; lane 7, 10%; lane 8, negative control; and lane 9, 1-kb DNA ladder. Lanes 4–7 contain DMSO in PCR step only. RNA template was added into the master mixture of the reaction and aliquoted for even distribution of template during RT.

APPENDIX 6

American Journal of Pathology, Vol. 148, No. 4, April 1996

Technical Advance

Triple Primer Polymerase Chain Reaction

A New Way to Quantify Truncated mRNA Expression

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The most practical method to quantify mRNA expression within small tumor samples is reverse transcription (RT) followed by quantitative polymerase chain reaction (PCR). One approach, known as "competitive RT-PCR" allows absolute quantitation by reference to synthetic RNA standards but is time-consuming and requires multiple manipulations that limit its usefulness as a screening assay. We describe here a new approach to quantify truncated type mRNAs relative to the wild-type transcripts in small amounts of tissue. This technique, called RT-triple primer-PCR, consists of coamplification of wild-type and truncated cDNAs using three primers in the PCR. To validate this approach, a truncated estrogen receptor variant (clone 4) was quantified relative to the wild-type estrogen receptor using plasmid preparations. The ratio of triple primer-PCR products obtained was directly related to the initial ratio of input cDNAs. RT-triple primer-PCR was then used to compare the relative expression of clone 4 mRNA in frozen sections of normal human breast tissue and human breast tumors with characteristics of good prognosis. The statistically significant difference (P = 0.03) observed between normal and tumor tissues suggests that elevated expression of the clone 4 variant may be associated with early steps of tumorigenesis. This technique provides a useful alternative to already described quantitative RT-PCR techniques for the quantification of truncated mRNA within small amounts of biological material. (Am J Pathol 1996, 148:1097–1103)

One manifestation of altered gene expression in many human diseases is the production of truncated or modified mRNAs that may be translated into modified proteins that act abnormally. Such mRNAs are involved in diseases as different as Glanzmann thrombasthenia,[1] the most common inherited disorder of platelets; aspartylblucosaminuria,[2] an inherited lysosomal storage disorder; Duchenne and Becker muscular dystrophies;[3] or cancer progression.[4,5] Several estrogen receptor (ER) variant mRNAs have also been identified in human breast cancer biopsies.[6–9] The knowledge of the relative proportion of these modified mRNAs to the wild-type mRNA can provide a useful tool in diagnosis, prognosis, or survey of the disease. The accurate quantification of such mRNAs is often difficult, and the more commonly used quantitative techniques such as Northern blot or RNAse protection assay are not sensitive enough to allow assessment of expression within small pathologically defined regions of tissue or small cell numbers. Reverse transcription associated with polymerase chain reaction (RT-PCR) is

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often the only method that can be used to qualitatively or quantitatively determine mRNAs when sample size and/or abundance are limiting factors.[10–12] "Comparative RT-PCR," which involves assessment by reference to a "housekeeping" gene, is often used but suffers from the assumption that the reference point is stable.[13] An alternative is "competitive RT-PCR," which involves the coamplification of a synthetic standard that is distinguishable from the cDNA to be quantified by the presence of an introduced restriction site or by differences in length.[14,15] This approach is based on the principle that the two targets will compete for the annealing of the two primers. Therefore, an equivalent signal after amplification means that the control DNA and the cDNA to be quantified are present in the same proportion. Techniques based on this approach have been successfully used by many laboratories. Theoretically, this approach will allow absolute quantitation, but this can be at the cost of multiple manipulations and large quantities of RNA, and therefore precious tissue samples. For these reasons we concluded that comparative and competitive RT-PCR techniques were not optimal for rapidly quantitating the relative expression of truncated mRNAs to wild-type mRNA in multiple small breast tissue samples. We therefore tested a new approach called RT-triple primer-PCR (RT-TP-PCR) to assess ER variant expression in microdissected human breast specimens.

To validate this assay, we chose to measure the relative expression of a truncated ER mRNA variant (clone 4) to the wild-type ER (WT-ER) mRNA. This truncated ER mRNA variant was initially characterized by sequencing a 2333 bp cDNA isolated from a human breast tumor cDNA library,[16] and was shown to present significantly elevated expression relative to the WT-ER transcript in tumors with parameters of poor prognosis and endocrine insensitivity.[17] As shown in Figure 1, clone 4 cDNA consists of sequences identical to exons 1 and 2 of the human ER, followed by sequences that are unrelated to those found in human ER cDNA. To perform TP-PCR, three primers are used. The upstream primer (E2U) recognizes both the truncated variant and the wild-type cDNAs. The two downstream primers (E3L, C4L) are specific for the WT cDNA and ER-clone 4 cDNA, respectively. Since the upstream primer can anneal to both cDNAs, TP-PCR leads to a competitive amplification of truncated and wild-type cDNAs, the final ratio between the coamplified products being related to the initial input cDNA ratio.

After validating this technique, it was used to compare the relative expression of clone 4 mRNA to WT-ER mRNA in normal human breast tissue and human breast tumors that displayed characteristics of good prognosis.

Materials and Methods

Human Breast Tissues and Cell Lines

Normal breast tissues were obtained from reduction mammoplasty specimens collected at the laboratory of F. Kuttenn of the Necker Hospital (four cases) and at the Manitoba Breast Tumor Bank (four cases). Human breast tumor specimens were obtained from the Manitoba Breast Tumor Bank (10 cases). All specimens had been rapidly frozen at −70°C as soon as possible after surgical removal. A portion of the frozen tissue from each case (normal and tumor) was processed to create formalin-fixed and paraffin-embedded tissue blocks, matched, and oriented relative to the frozen tissue. This allows high quality histological sections to be assessed and pathological interpretation of the corresponding frozen sections from the immediately adjacent frozen tissue block. The presence of normal ducts and lobules was confirmed in all normal tissue specimens, as well as the absence of any atypical lesion. The 10 primary ductal carcinomas were selected from the Tumor Bank on the basis of a set of several parameters that are indicative of a good prognosis. All tumors were well differentiated (Nottingham grade 4 or 5), ER- and progesterone receptor (PR)-positive as determined by ligand binding assay (ER >3 fmol/mg protein, PR >15 fmol/mg protein), and axillary node-negative. Specific frozen tissue blocks were chosen in each case on the basis of several further criteria as assessed in histological sections. These tissue criteria included: a cellular content of >30% invasive tumor cells with minimal normal lobular or ductal epithelial components, good histological preservation, and absence of necrosis.

Ishikawa cells, an endometrial adenocarcinoma cell line initially established by Dr. H. Iwasaki (Tsukuba, Japan), were provided by Dr. E. Gurpide (Mount Sinai School of Medicine, New York, NY). These cells are known to express different ER variant mRNAs (L. C. Murphy, unpublished data) and have therefore been used as positive controls. Cos-1 cells (American Type Culture Collection, Rockville, MD) do not express ER mRNA and were used as a negative control in our experiments. Cells were grown and harvested to obtain cell pellets, which were stored at −70°C, as previously described.[18]

Plasmids

The pHEGO plasmid contains the previously cloned and sequenced WT-ER cDNA and was kindly provided by P. Chambon.[19] Clone 4 plasmid contains the previously cloned and sequenced clone 4 truncated ER variant cDNA.[16] These two plasmids were mixed to obtain solutions in which the clone 4/WT-ER cDNA proportions ranged from 1/1000 to 1/1, while maintaining a total constant plasmid concentration of 0.1 ng/μl.

Extraction of mRNA and RT

Total RNA was extracted from histologically defined regions within 20 μm cryostat sections of frozen normal and tumor tissue using a small scale RNA extraction protocol (Trireagent, MRCI, Cincinnati, OH) according to the manufacturer's instructions. The yield was quantitated by spectrophotometer in a 50 μl microcuvette. The average yield of total RNA per 20 μm section was 4 $\mu g/cm^2$ for tumor and 0.6 $\mu g/cm^2$ for normal tissues (±20% variation with cellularity) with minimal contamination by DNA (optical density$^{260/280}$ > 1.8).

RT was performed in a final volume of 15 μl. RNA (600 ng) was reverse transcribed in the presence of 1 mmol/L deoxyadenosine-5′-triphosphate (dATP), 1 mmol/L deoxycytidine-5′-triphosphate (dCTP), 1 mmol/L deoxyguanosine-5′-triphosphate (dGTP), 1 mmol/L deoxythymidine-5′-triphosphate (dTTP), 5 mmol/L dithiothreitol (GIBCO-BRL, Grand Island, NY), 1 unit/μl ribonuclease inhibitor (Promega, Madison, WI), 20 μmol/L random primers, 50 mmol/L Tris-HCl (pH 7.5), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, and 5 units/μl Moloney Murine Leukemia Virus reverse transcriptase (GIBCO-BRL) for 10 minutes at 22°C and 1 hour at 37°C. After 5 minutes at 95°C, 1 μl of the reaction mixture was taken for subsequent amplification using PCR.

Primers and TP-PCR Conditions

Three primers were used in this study (Figure 1). E2U (5′-AGGGTGGCAGAGAAAGAT-3′, sense, located in WT-ER exon 2; 708–725) and E3L (5′-TCATCATTCCCACTTCGT-3′, antisense, located in WT-ER exon 3; 969–986) allowed amplification of a 281 bp fragment corresponding to WT-ER mRNA. E2U and C4L (5′-GGCTCTGTTCTGTTCCATT-3′, antisense; 941–959) allowed amplification of a 249 bp fragment corresponding specifically to clone 4 truncated ER variant mRNA. Positions given correspond to published sequences of ER cDNA[20] for E2U and E3L and of clone 4 cDNA[16] for C4L primer.

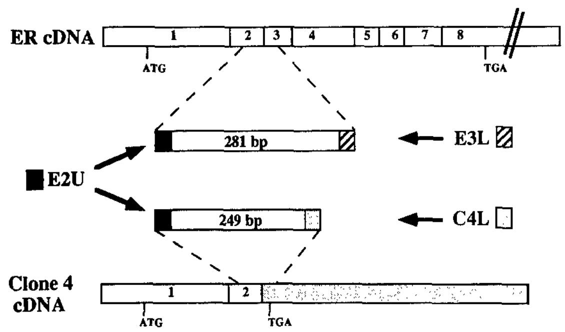

Figure 1. *Schematic representation of TP-PCR. Three primers are used simultaneously during the PCR. The upper primer (E2U) is able to recognize both WT-ER cDNA and truncated clone 4 cDNA. The lower primers (E3L and C4L) are specific for each cDNA. Competitive amplification of a 281 bp and a 249 bp fragment occurs, giving a final PCR-product ratio related to the initial input cDNA ratio.*

PCR amplifications were performed using 1 μl of RT mixture or plasmid solution in a final volume of 10 μl, in the presence of 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L dATP, 0.2 mmol/L dTTP, 0.2 mmol/L dGTP, 0.2 mmol/L dCTP, 4 ng/μl of each primer (E2U, E3L, and C4L), 1 unit of Taq DNA polymerase (GIBCO-BRL), and 10 nmol/L of dCTP [α-^{32}P] (ICN Pharmaceuticals Inc., Irvine, CA). Each PCR consisted of 40 cycles (1 minute at 60°C, 1 minute at 72°C, and 1 minute at 94°C) using a Thermocycler (Perkin-Elmer Cetus, Norwalk, CT). Three μl of the reaction was then denaturated in 80% formamide buffer and subjected to polyacrylamide gel electrophoresis (PAGE) on 6% gels containing 7 mol/L urea. After electrophoresis, the gels were dried and exposed to Kodak XAR Film at −70°C with two intensifying screens for 2 hours.

Quantification and Statistical Analysis

Autoradiographs were analyzed with a densitometry system based on a charge-coupled device camera (DAGE 72) and MCID M4 software (Imaging Research Inc., Sainte Catherines, ON). The signal corresponding to clone 4 was expressed as a percentage of the corresponding WT-ER signal; a value of 100% means that the clone 4 corresponding signal is equivalent to the wild-type corresponding signal.

For each sample, at least three independent measures of the clone 4 relative expression were performed and the mean determined. Means obtained from the eight normal breast samples were then compared with that found in the 10 tumor tissue samples using the Mann-Whitney rank sum test (two-sided).

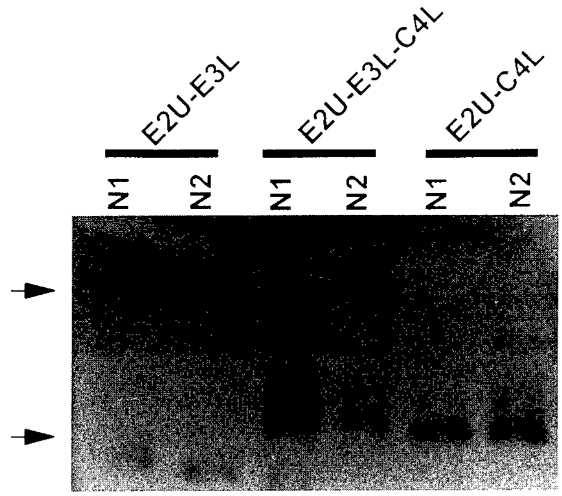

Figure 2. *Amplification of normal breast tissue cDNA using two or three primers in PCR. RNA from two normal breast tissue samples (N1 and N2) was reverse transcribed and amplified by PCR using E2U-E3L, E2U-E3L-C4L, or E2U-C4L primers. PCR products were separated by PAGE and analyzed as described in Materials and Methods. Upper and lower arrows show wild-type (281 bp) and clone 4 (249 bp) corresponding signals, respectively.*

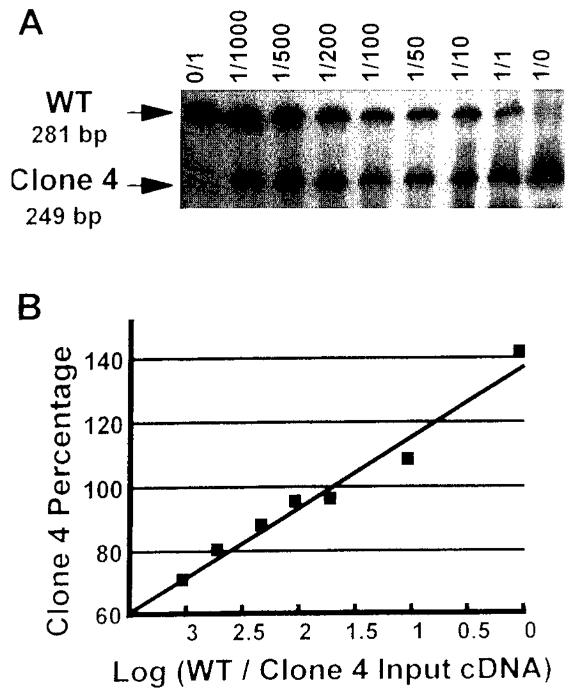

Figure 3. *Validation of TP-PCR technique using different plasmid preparations.* A: *Different plasmid preparations (0.1 ng) were amplified by TP-PCR: plasmid containing WT-ER cDNA alone (0/1), a mix of plasmids containing WT and truncated cDNA in varying ratios of clone 4/wild type ranging from 1/1000 to 1/1, or plasmid containing clone 4 alone (1/0). PCR products were separated by PAGE and analyzed as described in Materials and Methods.* B: *A mix of plasmids containing WT-ER and truncated clone 4 ER variant in varying proportions were analyzed by TP-PCR as described above. The percentage of clone 4 signal relative to the wild-type signal is expressed as a function of the log of WT/clone 4 input cDNA ratio.*

Results

Coamplification of WT-ER and Clone 4 Truncated ER Variant cDNAs Using TP-PCR

The ability of WT-ER and clone 4 cDNAs to be amplified simultaneously in a PCR reaction using three primers (TP-PCR) was initially determined. Total RNA from two normal breast tissue samples was analyzed by RT-PCR using E2U-E2L, E2U-C4L, or E2U-E3L-C4L primers (Figure 2). Using E2U and E3L, a band of 281 bp, corresponding to WT-ER cDNA was obtained. E2U and C4L primers allowed amplification of a 249 bp band, corresponding to clone 4 cDNA. When the three primers were present during the PCR reaction, both bands were obtained.

Ratio of the Clone 4 Signal Relative to Wild-Type Signal Is Constant and Proportional to the Initial Wild-Type/Clone 4 cDNA Ratio

The maintenance of a constant ratio of clone 4/WT-ER signals after RT-TP-PCR was examined under varying PCR conditions. To address this issue, RNA from Ishikawa cells, known to express clone 4 truncated ER mRNA (L. C. Murphy, unpublished data) was reverse transcribed and amplified using E2U-E3L-C4L primers for a varying number of cycles ranging from 20 to 45. Quantification of signals showed that the ratio clone 4/WT signals did not vary by more than 20% (data not shown). Similarly, using a constant number of PCR cycles (40 cycles), variation of input cDNA ranging from 50 to 0.1 ng did not affect this ratio by more than 20% (data not shown).

The relationship between the input ratio of clone 4/WT-ER cDNA and the clone 4/WT-ER signal ratio after TP-PCR was determined next. pHEGO and clone 4 plasmids, containing WT-ER cDNA and clone 4 cDNA, respectively, were mixed in varying proportion, ranging from 1000/1 to 1/1. TP-PCR was performed on these samples. Autoradiography showed that the intensities of the two PCR products are directly related to the initial ratio of cDNAs added (Figure 3A). Quantification of signals revealed a linear relationship between the final percentage of clone 4 and the log of the initial WT/clone 4 ratio (Figure 3B).

Comparison of Clone 4 Truncated ER Variant mRNA Expression in Normal Tissues and Tumor Tissues with Characteristics of Good Prognosis

The relative level of clone 4 variant ER mRNA expression was then measured by RT-TP-PCR in 8

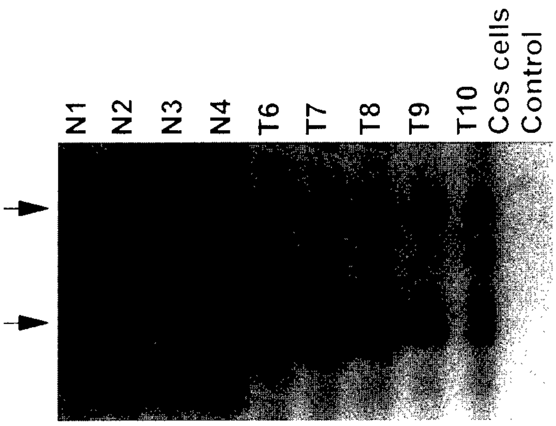

Figure 4. *Measurement of the relative expression of clone 4 variant ER mRNA in normal and neoplastic human breast tissues. RNA extracted from normal (N1–N4) or neoplastic (T6–T10) breast tissues was analyzed by RT-TP-PCR. PCR products were analyzed as described above. Negative controls consisted of RNAs from Cos-1 cells analyzed simultaneously (Cos cells), or no added cDNA in TP-PCR reaction (control). Upper and lower arrows show wild-type and clone 4 corresponding signals, respectively.*

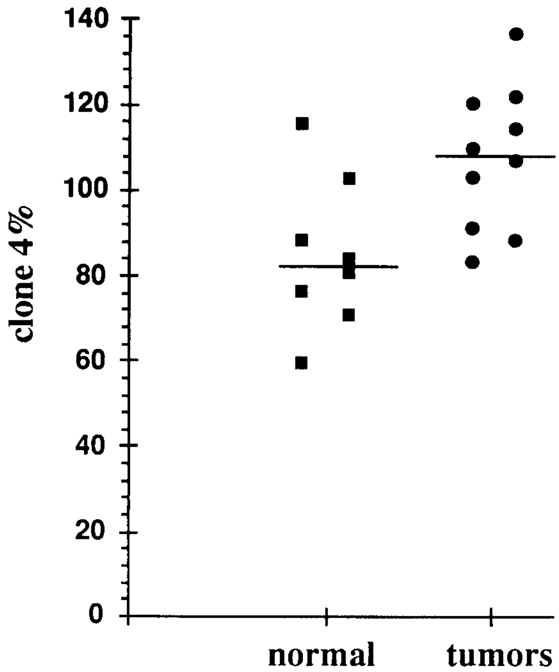

Figure 5. *Quantitative comparison of the relative expression of clone 4 variant ER mRNA in normal human breast tissue and human breast tumors with characteristics of good prognosis. RNAs extracted from 8 normal breast tissue samples and 10 tumors with characteristics of good prognosis were analyzed by RT-TP-PCR as described in Materials and Methods. For each sample, the mean of three independent measures of the clone 4 relative expression, expressed as a percentage of the corresponding wild-type ER signal, was determined (□, means of normal breast samples; ○, means of tumor breast samples). Bars represent medians.*

normal breast tissues and 10 breast tumors with characteristics of good prognosis (Figure 4). For each sample, the mean of three independent measures of the clone 4 relative expression, expressed as a percentage of the corresponding WT-ER signal, was determined (Figure 5). Using the Mann-Whitney rank sum test, the relative expression of clone 4 truncated variant ER mRNA to WT-ER mRNA was found to be significantly ($P = 0.03$) lower in normal breast (median = 82.5%) *versus* neoplastic breast tissues (median = 107.5%).

Discussion

In this manuscript, we provide strong evidence that TP-PCR is a reliable quantitative technique to determine relative expression of truncated transcripts, the percentage of signals measured after TP-PCR being directly correlated to the initial input ratio. This new PCR-based quantification provides several advantages over existing techniques. There is no need for synthetic controls, the internal control for both RT and PCR amplification being provided by wild-type mRNA. Moreover, serial dilutions are not necessary, and the requirement for only one tube decreases the risk of contamination and degradation as well as decreasing variability associated with multiple pipettings. This technique is optimal for the study of multiple tissue samples where the quantity of material is limited. During the preparation of this manuscript, a similar approach was used to coamplify wild-type glucocorticoid receptor and a truncated variant in myeloma patients.[21] However, in this study, the technique was used to compare relative expression, and the quantitative nature of this PCR approach was not tested.

TP-PCR was applied to assess the clone 4 truncated ER variant mRNA expression in normal and neoplastic human breast tissue. Several ER variants have previously been identified in breast cancer biopsies and cell lines.[6–8] Some of the ER-like proteins encoded by these variant mRNAs lack some ER functional domains, and have been shown to exhibit altered functions or interfere with WT-ER function.[8,22,23] Therefore, it has been speculated that these ER variants may be involved in progression from hormone dependence to independence in breast cancer.[24] Many of these variant ER mRNAs have now also been detected in normal breast tissue.[25,26] This suggests that variant ER-like proteins may play a role in the normal ER signaling pathway and that any change in the relative proportion of these variants could therefore lead to deregulation of this pathway, which may contribute to tumorigenesis. Some support for such an hypothesis had been ob-

tained previously when we used an RT-PCR approach to show that the relative expression of exon 5-deleted ER mRNA to wild-type transcript was significantly higher in breast tumors compared with normal breast tissue.[25] Furthermore, we had previously shown, using an RNAse protection assay, that clone 4 truncated mRNA was significantly elevated relative to WT-ER mRNA in those breast tumors which had characteristics of poor prognosis and hormone independence.[17] Using RT-TP-PCR, we report here that the expression of clone 4 truncated ER mRNA is significantly lower in normal tissues compared with human breast tumors with characteristics of good prognosis (ER^+, PR^+, node-negative). This result, together with our previous data, strongly supports the hypothesis that deregulation of ER variant mRNA expression occurs at relatively early steps in human breast tumorigenesis, and may indeed have a role in this process.

RT-TP-PCR, like any gel-based technique for RNA assessment (Northern blot, RNAse protection assay or RT-PCR) does not give information about the cell to cell pattern of expression. The determination of the cellular origin of expression requires the combined use of an *in situ* type technique such as *in situ* hybridization or immunohistochemistry. This technique provides, however, a useful preliminary screening method for evaluating the variant mRNAs expression. TP-PCR can be adapted to the study of numerous biological problems involving variant mRNA containing unique sequences linked to the sequences shared with wild-type transcripts. Apoptosis involving several partners, including *Bcl-2*, *Bcl-x* and bax, provides an example where relative expression of such variant mRNAs could be explored using TP-PCR.[27,28] Baxβ mRNA effectively contains unspliced sequences absent from baxα mRNA. The short form of the orphan receptor FTZ-F1 that was recently found to regulate the wild-type protein activity[29] is coded by a variant mRNA, the expression of which could also be explored by this technique. TP-PCR can also be adapted to DNA studies. Quantification of translocated DNA regions relatively to the wild-type DNA could allow the estimation of an abnormal cell population relative to the normal one in leukemia patients. TP-PCR can therefore provide a useful tool in diagnosis, prognosis, or survey of numerous diseases.

Acknowledgments

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APPENDIX 7

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<ABSTRACT>The evolution of breast cancer into an estrogen-independent growth phenotype marks the beginning of a more aggressive phase of the disease and is a major problem in the efficacy of endocrine therapies. In some cases, hormone-independence and resistance can occur due to loss of estrogen receptor (ER) expression, but at least 50% of tumours which have developed resistance to endocrine therapy remain receptor positive. T-47D5 human breast cancer cells are ER+ and estrogen treatment in culture results in increased proliferation of these cells. An estrogen-nonresponsive cell line (T5-PRF) was developed from T-47D5 cells, by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen while still retaining expression of the ER. Transient transfection studies have been performed using an estrogen-responsive reporter gene system. In the absence of ligand T5-PRF cells have ~4 fold increased basal ER activity compared to the parent T-47D5 cells. Long range ER RT-PCR has also been performed to characterize the pattern of variant ER mRNA expression between the two cell lines and differential expression of ER mRNA variants was found. In particular, an ER variant mRNA, deleted in exons 3 and 4, was detected only in T5-PRF cells. These results suggest that defects in the ER structure and function, or activation of ER via ligand-independent mechanisms may underly hormone-independence in breast tumours.

cells themselves (4.0%). Of 75 informative cases, 2.6% expressed no IGF-II mRNA, 46.7% had low levels of expression, 26.7% had moderate levels and 24.0% had high levels of expression. There was good concordance between mRNA and protein levels (75%). IGF-II expression did not correlate T or N stage, nuclear grade, p53 or overall survival. Furthermore, IGF-II expression correlated with poor survival in ER- patients ($p = 0.007$) and PR- patients ($p = 0.01$). Patients who were ER(+) IGF-II(+) had disease free survival of 85% while patients who were ER(−) IGF-II(+) had disease free survival of just 40%. We conclude that IGF-II expression is a frequent event in breast cancer and its expression may predominantly exert a paracrine effect on breast epithelium. Interestingly, high levels of IGF-II may be associated with a poor outcome in those patients who are ER- and PR-.

#1640 Sunday, April 21, 1996, 8:00–12:00, Room 30

Amphiregulin and cripto-1 activity in the mouse mammary gland. Kenney, N.J., Smith, G.H*., Salomon, D.S*. and Robert B. Dickson. *Lombardi Cancer Center, Georgetown University, Washington DC, and *National Cancer Institute, NIH, Bethesda, MD.*

As the juvenile mouse mammary gland matures it undergoes extensive epithelial proliferation leading to a network of ductal branching which transverses the organ. Recent evidence suggests that the epidermal growth factor (EGF) family of peptides amphiregulin (AR), cripto-1 (CR-1) may play multiple roles in the proliferation, differentiation and neoplastic conversion of the mouse mammary gland. Using a dual approach of recombinant AR and CR-1 in slow-release pellets and overexpression cDNA *via* retroviral technology, we have explored the biogenetic activity of each of these genes in the mouse mammary gland. Thus far, our observations indicate that recombinant AR and CR-1 can reestablish longitudinal ductal proliferation in growth quiescent mammary glands of ovariectomized mice. Further, in separate experiments, transduced mammary transplants overexpressing AR and CR-1 resulted in hyperplastic tertiary ducts, hyperplastic lobules and increased lateral branching which were apparent after 9 weeks. We suggest, that specific members of the EGF family can reestablish the early developmental activity of hormonally-deprived ductal mammary epithelium and induce hyperplasia *in vivo* which may further implicate this family as an important intermediary in epithelial maturation and early malignant progression within the mammary gland.

#1641 Sunday, April 21, 1996, 8:00–12:00, Room 30

Heregulins and mammary ductal morphogenesis. Nicholas J Kenney, Dajun Yang, Marc Lippman and Robert B. Dickson. *Lombardi Cancer Center, Georgetown University, Washington DC.*

Development and maturation of mammary epithelium depends on growth factors through a highly complex series of events. Recently, several members of the EGF growth factor family have been implicated in this process. Heregulins (HRG) alpha and beta, newly described members of this family have been shown to stimulate mammary morphogenesis *in vitro* and stimulate the phosphorylation of the erbB3 and erbB4 receptors in human breast cancer cells lines. We have generated several recombinant isomers (extracellular domain only) of the HRG family and tested these proteins for bioactivity *in vivo* by utilizing slow release pellets implanted into the #4 mammary glands of 5 week old ovariectomized C57B16 mice. Our results indicate that 5–7 ug/pellet of HRGα after 5 days of treatment initiated lateral and longitudinal ductal branching similarly observed in pellets containing 5–7 ug/pellet of EGF. In addition, we also observed that HRGα induced an increase in ductal width as well as ductal migration towards the pellet compared to control BSA pellets. These observations support previous evidence of HRG bioactivity in mammary cultures *in vitro* and suggest that HRGs may also contribute to the growth and maintenance of normal and malignant mammary epithelium *in vivo*.

#1642 Sunday, April 21, 1996, 8:00–12:00, Room 30

Differential display reveals a gene involved in breast cancer. A. Hornby[1], M. McBride-Putman[2], S. Fuqua[2], R. Lupu[1]. [1]*Georgetown University, Washington, D.C. and* [2]*University of Texas, San Antonio, Texas.*

Growth factors and their receptors play an important role in breast cancer. The ligand, heregulin, stimulates tyrosine phosphorylation of the receptors erbB3 and erbB4 directly and erbB2, indirectly. Furthermore, MCF-7 cells transfected with this ligand (MCF-7/T) become estrogen-independent, grow in soft agar and grow large tumors in nude mice in the absence of estradiol, in contrast to wild-type MCF-7 cells (Tang et al., 1994 Proc. AACR:35, p36). In an attempt to understand the function of heregulin, differential display PCR was performed and a clone isolated based on the comparison of MCF-7/wt cells with MCF-7/T cells. This clone, DDclone2, is expressed in heregulin-transfected cells (MCF-7/T) and not in wild-type MCF-7 cells (MCF-7/wt). A panel of breast cancer cell lines was examined by Northern blot analysis for the expression of heregulin and DDclone2. While heregulin expression was seen in the aggressive cell lines, MDA-MB-231, HS578T, BT549, and MCF-7/T, DDclone2 expression was seen in BT549 and MCF-7/T cells. Three transcripts were visible at 2.5, 2.0 and 1.7 kb. DDclone2 was sequenced and appears to be a novel gene. We are currently cloning the full-length cDNA, the sequence of which may give some clue as to its function and direct us in future experiments.

#1643 Sunday, April 21, 1996, 8:00–12:00, Room 30

Estradiol-17β (E_2)-regulated expression of protein tyrosine phosphatase gamma (PTP-γ) gene in cultured human breast and breast cancer cells. Zheng, J.[1], Zhang, Y.[1], Sugimoto, Y.[1], Mulla, Z.[1], Canatan, H.[1], Dayton, M.A.[2], Govindan, M.V.[3], Farrar, W.B.[1], Brueggemeier, R. W.[1], and Lin, Y.C.[1] [1]*The Ohio State Univ., Columbus, Ohio 43210,* [2]*Louisiana State Univ., Shreveport, LA 71130,* [3]*Laval Univ., Quebec, Canada.*

PTP-γ is a potential tumor suppressor gene in human kidney and lung cancers. Our previous results have also shown that PTP-γ mRNA is expressed in both primary cultured human breast cells and breast cancer cell lines by RT-PCR. By RNase protection assay, we found that PTP-γ gene expression level was lower in human breast cancer cells than that in the normal breast cells. We also demonstrated that PTP-γ mRNA expression was inhibited by E_2 dose-dependently in primary cultured normal breast cells. After the cells were treated with 20nM of E_2 for 24 hours, PTP-γ mRNA expression was significantly inhibited in both primary cultured cancerous and non-cancerous cells from breast cancer patients, as well as in estrogen receptor (ER)-positive MCF-7 cell line by 50%, 85%, and 66%, respectively. However, the expression level did not change in the ER-negative MDA-MB-231 cell line. PTP-γ mRNA expression was significantly inhibited (by 94%) in ER-transfected MDA-MB-231 transfectants which was transfected with an ER expression plasmid. On the other hand, 1 μM of progesterone significantly stimulated PTP-γ mRNA expression by 7-fold. Our results showed that estrogen significantly inhibited PTP-γ gene expression in cultured human breast cells and this inhibition is mediated by ER, while progesterone significantly stimulated it. Our results is the first to suggest that PTP-γ is a potential estrogen-regulated tumor suppress gene in human breast cancer and may play an important role during neoplastic processes in the human breast. (Supported by NIH grants DK45916, CA58003 and CA66193.)

#1644 Monday, April 22, 1996, 1:00–5:00, Poster Section 9

Leukemia inhibitory factor (LIF) and its receptor (LIFR) in breast cancer: a potential autocrine/paracrine growth regulatory mechanism. Dhingra, K., Sahin, A., Emami, K., Estrov, Z. *M.D. Anderson Cancer Ctr, Houston, TX.*

Human breast tumors show a high propensity to metastasize to the bone/bone marrow. This may be due to the growth stimulatory effect of hematopoietic cytokines on breast tumor cells. We have recently shown that cultured human breast cancer cells express LIFR and can be stimulated to grow by LIF (which is known to be constitutively produced by bone marrow stromal cells) (J. Interferon Cytokine Res., 15:905, 1995). To investigate the *in vivo* relevance of these observations, we developed an immunohistochemical staining method for LIFR using a monoclonal antibody M1 (kindly provided by Bettina Thoma, Immunex, Inc.). LIFR expression was detected in 79% (38/48) specimens. In 29 specimens, the expression was detected at a high frequency (50–75% positive cells-4 specimens; 75–100% positive cells-25 specimens). Interestingly, the majority (80%) of these specimens also showed positive LIF immunostaining (using a monoclonal antibody D62.3.2, kindly provided by K. Jin Kim, Genentech, Inc.). LIFR positivity was higher in well-differentiated tumors ($p = 0.04$) but expression of LIF/LIFR did not correlate with other conventional prognostic features. These findings support a potential role for LIF/LIFR in autocrine/paracrine growth regulation of breast cancer.

#1645 Monday, April 22, 1996, 1:00–5:00, Poster Section 9

Expression of tumor markers during breast tumor progression. Wani, G., Noyes, I., Milo, G.E., and D'Ambrosio, S.M., *Depts. Radiology. & Medical Biochemistry, The Ohio State University, Columbus, OH 43210.*

Surgical specimens of breast tubular ductal carcinomas, and invasive ductal carcinomas, were grafted onto 3–4 wk old female nude mice. Fresh tumor tissue and progressively growing tumors were harvested every two wk and analyzed immunohistochemically. The expression of molecular biomarkers (estrogen receptor, p53, CerbB-2, and cyclin D1) associated with the aggressive nature of the tumor were characterized as a function of surrogate tumor progression and compared to the fresh tumor. The data indicates that: (1) in tubular ductal carcinomas the diversity of cellular morphology is conserved in the xenograft. (2) A strong specific staining of ductal cells compared to other structures in both the tumor and progressively growing surrogate tumor was observed for the estrogen receptor, cyclin D1, and CerbB-2. (3) A diffused and heterogeneous staining was observed for p53. These spatial areas of molecular biomarker expression observed in both the fresh and surrogate tumors were not apparent in normal breast tissue. These data support the tenet that the diversity of cellular morphology and differential spatial expression of molecular biomarkers is consistent with the proposal that the heterogenous cellular matrix of the breast tumor indicates that there are diverse phenotypes present during the different stages of the progression in both the tumor and surrogate tumor. (Supported by NCI grant P20-66193.)

#1646 Monday, April 22, 1996, 1:00–5:00, Poster Section 9

Quantitation of estrogen receptor clone 4 mRNA variant in microdissected normal and neoplastic human breast tissues. Hiller, T.[1], Leygue, E.[2], Murphy, L.[2], Watson, P.H.[1]. *Departments of Pathology*[1] *and Biochemistry & Molecular Biology*[2], *University of Manitoba, Winnipeg, Canada. R3E OW3.*

Estrogen receptor (ER) mRNA variants may play a role in progression in human breast cancer and we have also found that the expression of ER deletion 5 variant

) wild type ER (ER wt) can differ between normal and neoplastic breast tissue :t al, JNCI, in press). To pursue the role of 'truncated' and 'deleted' ER mRNA in the development of breast cancer further we have developed a novel to facilitate microdissection and RNA extraction from small pathologically egions within frozen breast tissue sections. Histologically defined areas less n² can be microdissected to provide an average yield of 1.0 μg (+/− 0.5 μg) RNA from 5 serial 20 μm sections. We have also developed a specific mer RT-PCR assay to assess the expression of the truncated ER clone 4 ıriant relative to the wt ER based on internal competition between 3 primers. nary study of unmatched samples showed a significant increase in the level of 4 in tumors relative to normal (ER clone 4/ER wt, median tumor level = ı = 10; median normal level = 82.5%, n = 8; p = 0.03). We have now begun our analysis of ER clone 4 and ER deletion variant expression to regions of yperplastic, in-situ and invasive components within single tumor tissue sec- ıported by Canadian Breast Cancer Initiative and USAMRDC.

Monday, April 22, 1996, 1:00–5:00, Poster Section 9

ive reverse transcription polymerase chain reaction analysis of c-myc n in breast cancer. Singh R., & Watson P.H. *Dept of Pathology, University ba, Winnipeg, Manitoba, Canada. R3E OW3.*

on of c-myc gene expression has been implicated in the progression of breast l the transition to hormone independent growth. As a first step to determine l relevance, we have studied the effect of specimen collection time of tumors :l of c-myc expression. We have developed competitive RT-PCR assays using cRNA standards to quantitate c-myc mRNA and estrogen receptor (ER) tracted from small microdissected breast tumor samples. The c-myc assay ırately distinguish a minimal 2-fold difference by comparison with Northern ıe ER assay could distinguish a 100 fold difference in ER level in mixtures and ER−ve breast cell lines. We then assessed the relatively unstable c-myc l the stable ER mRNA in multiple homogeneous samples collected from 10 or surgical specimens and stored on ice for 0, 3, 6 and 24 hours prior to ıd subsequent RNA extraction. The levels of c-myc declined over 24 hours in (mean of 74% of the level at time 0, sd 77%, max 31%, min 93%, n = 10) levels showed smaller reductions (mean 95%, max 91%, min 97%, n = 4). rences in the rate of c-myc decline were independent of the tumor pathology adjacent tissue blocks. We conclude that knowledge of the time of collection specimens may be an important parameter to assess the role of alteration of NA gene expression (supported the Canadian Breast Cancer Initiative).

Monday, April 22, 1996, 1:00–5:00, Poster Section 9

ion of Genes Differentially Expressed in MCF-7 Breast Carcinoma Cells ıtment with 17 β-Estradiol. Go, V. and Pogo, B. G.T. *Mount Sinai School ?, New York, NY 10029*

ıan breast carcinoma cell line MCF-7 produces tumors with an increased and metastatic potential in athymic mice upon the co-introduction of 17 (Shafie and Liotta, Cancer Letters 11:81–87, 1980). Furthermore, the an estrogen-insulin axis adds to the complexity of unraveling the molecular s behind estradiol induced mammary tumor growth.

o determine the genes involved in increased tumor growth and metastasis as :stradiol induction, we have extracted total RNA from MCF-7 cells supple- h insulin along with or without 17 β-estradiol treatment. The differential ınique was implemented resulting in 91 possible differentially expressed ments of which 21 were found to be real. Northern analysis was used to ıe differences, and the differentially expressed fragments were cloned and evealing unique estradiol induced sequences.

Monday, April 22, 1996, 1:00–5:00, Poster Section 9

d hormonal/genetic pathways to breast cancer. Devra Lee Davis*⁺, H. ɔw⁺, Jack Fishman⁺, Michael Osborne⁺, Nitin Telang⁺. **World Re- itute, 1709 New York Avenue N.W., Washington, DC 20006, ⁺Strang cer Research Laboratory, Cornell University Medical College, New York,*

germ line mutations, such as loss of BRCA1 or AT, appear to account for ? of all breast cancer. Postnatal perturbations in the genome appear to be most cases of the disease. Cumulative lifetime exposure to bioavailable :s most known risk factors for breast cancer, excepting radiation. Based on :cent experimental and epidemiologic findings, we hypothesize that endo- ogenous hormones exert bi-functional biologic effects on hormonal and/or s to breast cancer. Depending on the periods and extent of exposure, the ıicologic consequences can be chiefly genetic, epigenetic, or hormonal. :-pubescent, or adolescent exposures to some xenohormones can work ırough genetic or epigenetic mechanisms chemically imprinting developing dify DNA structure or function, affecting replication, repair processes, and ion. Functional damage can affect such processes as phosphorylation of ., RB and phosphatidylinositol-3-kinase. This impedes critical cell repair dering recognition of damaged cells and allowing the accumulation of harmful mutations that activate cell cycle genes which can cooperate in the development of breast cancer. Structural damage to the DNA backbone can occur from direct genotoxic and from redox cycling of harmful xenoestrogens that produce reactive oxygen species, yielding high rates of radical-induced DNA damage, including 8-hydroxy-guanine and adenine adducts, apurinic sites, or loss of ring opening products (e.g., 2-6-diamino-4-hydroxy-5-formamidopyrimidine). Beneficial xenoestrogens, such as genistein and other bioflavonoids, may serve as antitoxidants or kinase inhibitors which either reduce aberrant breast cell proliferation and/or increase the rate of DNA repair. Adolescent, mid-life and post-menopausal exposures to some xenoestrogens, including anti-androgens, can be chiefly hormonal in action, working through two distinct mechanisms, which can influence the potential for aberrant cell growth: they can directly bind with the estrogen receptor increasing cell proliferation, or they can modify breast cell proliferation by altering hormonal metabolites that influence growth factors in other ways. Many synthetic xenoestrogens do not appear to bind SHBG, therefore their effective biologic activity will be higher than would be predicted from their concentrations. Once validated with in vitro and in vivo studies, biologic markers of the risk of breast cancer, such as hormonal metabolites, total bioavailable estradiol, and free radical generators, can be used to enhance the ability to prevent the disease. Biologic markers could also prove valuable as screening methods to identify persons at risk of developing the disease, as prognostic markers of those already diagnosed, as baselines against which to assess therapeutic interventions, and as experimentally based screening methods to assess new and existing chemicals, pharmaceuticals, and other widely used agents. Nutritional/therapeutic interventions can be devised that reduce the risk profile by increasing DNA repair capacity, antioxidant levels, or improving hormonal metabolism.

#1650 Wednesday, April 24, 1996, 1:00–5:00, Poster Section 1

Ovarian Sertoli-Leydig cell tumor represents a SRY gene independent pathway of "male" gonadal differentiation—report of a case with histogenetic considerations. Hittmair A., Zelger B.G., Obrist P., Rogatsch H., Dirnhofer S. *Department of Pathology, University of Innsbruck, Austria 6020.*

Sertoli-Leydig cell tumors (SLCT) are rare sex cord stromal tumors of the ovary generally composed of Leydig cells, gonadal stroma cells, and Sertoli cells resembling fetal testicular tubules. The histogenetic basis of morphological male differentiation patterns in a female organism has been a matter of debate for several years. Here, we report on a case of a SLCT with intermediate differentiation in a 23-year-old woman extensively investigated by light and electron microscopy, immunohistochemistry for intermediate filaments and sex steroid hormone receptors, as well as by polymerase chain reaction for the presence of the sex determining region Y (SRY) gene. As expected, polymerase chain reaction revealed lack of the SRY gene in SLCT derived genomic DNA. Immunohistochemistry showed strong expression of progesterone and androgen receptors as well as cytokeratins and vimentin in tumorous Sertoli cells. Electron microscopy rendered no Sertoli cell specific ultrastructural features. We conclude that SLCT represents a SRY gene independent pathway of "male" gonadal differentiation. The strong expression of progesterone receptors is in strict contrast to testicular Sertoli cells but constant with ovarian granulosa and surface epithelium cells. The results of the present investigation provide strong evidence for a very close histogenetic relationship between SLCT and granulosa cell tumor.

#1651 Wednesday, April 24, 1996, 1:00–5:00, Poster Section 1

Stability of the epithelial phenotype in human ovarian surface epithelium (OSE): an indicator of autonomy in ovarian carcinogenesis. Dyck, H.G.[1], Lynch, H.T.[2], Hamilton, T.C.[3], Godwin, A.K.[3], Maines-Bandiera, S.L.[1], Schultz, D.C.[3], Auersperg, N.[1] *[1]Obst./Gyn. Dept. Univ. B.C. Vancouver, V6H 3V5 Canada, [2]Creighton Univ. Omaha NE 681178, [3]Fox Chase Center, Phila. PA 19111.*

The OSE is the source of epithelial ovarian carcinomas. It is mesodermally derived and, in culture, converts to a mesenchymal phenotype by passage (p.) 3–6. In contrast, ovarian carcinomas express complex epithelial characteristics in vivo and in culture, suggesting loss of responsiveness to signals inducing mesenchymal conversion. To examine the onset of increased epithelial stability in neoplastic progression, OSE from 13 women with family histories (FH) and 25 women with no family histories (NFH) of ovarian and breast cancer, in low passage and SV40-immortalized, were compared to 8 ovarian cancer lines. By p.3–6, the % of cells positive for the epithelial markers keratin and CA 125 was lower in NFH-OSE than in FH-OSE cultures (p<0.05), whereas the % positive for the mesenchymal marker collagen 111 was higher in NFH-OSE (p<0.05). The immortalized and cancer lines retained keratin in >80% cells, and most cancer cells also expressed CA125 but lacked collagen. In 3-dimensional sponge culture NFH-OSE cells appeared mesenchymal and secreted extracellular matrix. FH-OSE cells were cuboidal without matrix, while the cancer lines formed epithelial layers, cysts and aggregates. Sponge contraction (a mesenchymal characteristic) was greater in NFH-OSE than in FH-OSE, both before and after immortalization, and absent in the cancer lines. Thus, increased stability of the epithelial phenotype is already evident in immortalized, nontumorigenic OSE, and in overtly normal OSE of women with histories of familial breast/ovarian cancer.

APPENDIX 8

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 228, 63–68 (1996)
ARTICLE NO. 1616

Identification of Novel Exon-Deleted Progesterone Receptor Variant mRNAs in Human Breast Tissue

Etienne Leygue,[1] Helmut Dotzlaw, Peter H. Watson,* and Leigh C. Murphy

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Received August 12, 1996

Using an approach based on the co-amplification of wild-type and exon deleted progesterone receptor (PR) variant cDNAs, we identified exon-deleted PR variant mRNAs in both normal and neoplastic breast tissues. Several naturally occuring variants, whose sequences revealed precise whole exon deletions, may encode putative PR-like proteins which lack some functional domains of the wild-type PR molecule. We suggest that these PR variant proteins could have a pathophysiological role in progestin action, as suggested for estrogen receptor variant proteins.

The progesterone receptor (PR), which belongs to the superfamily of ligand-activated nuclear transcription factors (1), is essential for progestin action in target tissues such as the endometrium and mammary gland. PR is an important prognostic marker in breast cancer as well as a marker of responsiveness to endocrine therapies (2). Its presence in estrogen receptor (ER) positive breast tumors generally indicates a high likehood of responsiveness to endocrine agents (3-4). In contrast, PR absence is often associated with failure to respond to these agents (5). Like all other members of the steroid/thyroid/retinoic acid receptor superfamily (6), PR is divided into structural and functional domains (A-E, Figure 1). Upon binding of ligand, PR dimerizes, undergoes phosphorylation and binds to specific sequences (PRE) located in the 5′ flanking region of PR-responsive genes (7). Further rounds of phosphorylation depending on DNA binding are also involved in trancriptional activation of the PR (8). Depending on the ligand, the isoforms involved (PR-A or PR-B), the target cell type, and the targeted gene, PR-activation will result in increased or decreased gene transcription (8-11).

Two functionally different PR isoforms, PR-A and PR-B (769 and 933 amino acids, respectively), have been previously identified in both normal and neoplastic human tissues (12). These two PR isoforms differ only in that PR-A lacks the NH2-terminal 164 amino acids of PR-B. PR-A and PR-B are translated from two distinct groups of mRNAs transcribed from the same gene under the control of two different promoters (Figure 1, 13). A third PR isoform (PR-C), that would be encoded by mRNAs lacking the translational start sites of PR-B and PR-A mRNAs but whose exact amino acid composition has not yet been established, has also been described (14). Beside the *M*r 90,000 PR-A, *M*r 120,000 PR-B and *M*r 60,000 PR-C proteins, several other PR-related proteins have been observed by Western blot in human breast tumors (15) and in T47D breast cancer cells (14). Characterization of these PR-related proteins and their possible significance in progesterone action still remain unclear. Furthermore, several PR-related mRNAs, ranging in size from 11.4 kb to 2.5 kb, as determined by Northern blot analysis, have been observed in both normal and neoplastic human tissues (16, 17). The origin of all these mRNAs remains unknown, although alternative promoter usage, alternative polyadenylation site selection and absence of

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Abbreviations: PR, progesterone receptor; ER, estrogen receptor; WT, wild-type; PCR, polymerase chain reaction.

0006-291X/96 $18.00

splicing have been suggested (17, 18). Although several of these mRNAs could encode PR-A and PR-B, other transcripts could not encode either of these isoforms (17, 18). The significance of the diversity of PR transcripts is therefore unclear. By analogy to the human ER and other members of the steroid receptor superfamily, we hypothesized that the diversity in PR-related transcripts could partly result from differential splicing. Several exon-deleted or truncated ER variant mRNAs have been observed in both normal and neoplastic tissues (19-24). The altered expression of some of these ER variant mRNAs and possibly the putative proteins encoded by these ER variant mRNAs, that lack some of the wild-type (WT) ER functional domains, has been suggested to be involved in the hormone independent phenotype of some breast tumors as well as in breast tumorigenesis (5, 24-28). It was therefore of interest to determine if similar exon-deleted PR variant mRNAs could also be observed in human breast tissues.

MATERIALS AND METHODS

Human breast tissues. Human breast tumor specimens (24 cases) and normal breast tissues obtained from reduction mammoplasty surgical specimens (10 cases), were collected at the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The presence of normal ducts and lobules was confirmed in all normal tissue specimens, as well as the absence of any atypical lesion. The twenty four primary invasive ductal breast carcinomas were associated with ER levels ranging from 0.5 to 386 fmol/mg protein, as determined by ligand binding assay . Within this group, 11 tumors were progesterone receptor positive (PR>15 fmol/mg protein), 12 were borderline positive (<15 fmol/mg protein) and 1 was PR negative (PR=0 fmol/mg protein), as also determined by ligand binding assay. The breast cancer cell line T-47D-5 was kindly provided by Dr. RL Sutherland (Garvan Institute for Medical Research, Sydney, Australia). These cells were previously shown to contain a high level of PR mRNA (29) and have therefore been used as positive controls. Total RNA was extracted and reverse transcribed in a final volume of 15 μl as previously described (24).

Polymerase chain reaction (PCR) and identification of PCR-products. The primers used consisted of PRU2 primer (5'-CCAGCCAGAGCCCACAATACA-3'; sense; located in PR exon 2; 2395-2415) and PRL2 primer (5'-GCAGCAATAACT-TCAGACATC-3'; antisense; located in PR exon 8; 3487-3467). The nucleotide positions given correspond to published sequences of the human PR cDNA (13). PCR amplifications were performed and PCR products analyzed as previously described (24). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 10 μl, in the presence of 10 nM [α-^{32}P] dCTP, 4 ng/μl of each primer and 1 unit of Taq DNA polymerase. Each PCR consisted of 40 cycles (1 minute at 60°C, 2 minutes at 72°C and 1 minute at 94°C). PCR products were then separated on 4% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and autoradiographed. In order to control for errors in input of cDNA used in PCR reactions, amplification of the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed in parallel and PCR products separated on agarose gels stained with ethidium bromide as previously described (24). PCR products were subcloned and sequenced as previously described (24). Predicted molecule mass and isoelectric point of the putative proteins encoded by the PR variant mRNAs identified in this study were obtained using MacVector™ 4.1.4 software (Kodak Scientific Imaging System, New Haven, CT).

RESULTS

Co-amplification of wild-type and exon-deleted PR mRNAs in breast samples. We used an approach adapted from a recently developed strategy used to study the prevalence of ER variant mRNAs within tumor samples (30). This approach is depicted in Figure 1. cDNAs corresponding to exon-deleted PR variants could be amplified together with the WT-PR mRNA using primers annealing with exon 2 (PRU2) and exon 8 (PRL2) sequences. In order to amplify variant mRNAs possibly related to both PR-A- and PR-B mRNAs, we have confined our approach to the region within exon 2 and exon 8, shared by these two mRNAs. Ten normal breast tissue samples obtained from reduction mammoplasties, and 24 breast tumor samples with a wide range of ER and PR levels, were studied. Total RNA was extracted from each sample, reverse transcribed and PCR performed in the presence of radiolabelled nucleotide. Figure 2 shows typical results obtained. Several different PCR products were observed in both normal and tumor samples. Three bands, that migrated with the apparent sizes of 1093 bp, 966 bp and 794 bp were observed reproducibly (i.e in at least two independent experiments) in most normal and neoplastic breast tissue samples, although the relative abundance of the bands seemed to differ amongst samples. These same bands were also observed in the PR positive T47D-5 human breast cancer cell line. Following subcloning

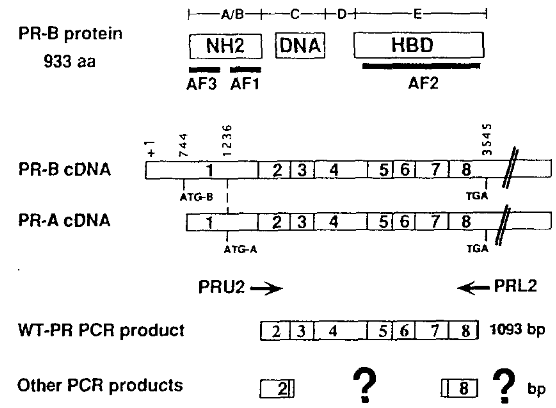

FIG. 1. Schematic representation of PR-B protein, PR-A and PR-B cDNAs, and primers allowing co-amplification of exon-deleted PR variant cDNAs. PR cDNAs contain 8 different exons coding for a protein divided into structural and functional domains (A-E). The NH2-terminal region A/B of the receptor contains two transactivation functions (AF3 and AF1). The DNA-binding domain of the receptor is located in the C region. Region D corresponds to the hinge domain of the protein and region E is involved in hormone binding and contains another transactivation domain (AF2). ATG-B and ATG-A are the translational start sites of PR-B and PR-A, respectively. PRU2 and PRL2 primers allow amplification of a 1093 bp fragment corresponding to WT-PR mRNAs. Co-amplification of all possible exon-deleted variants that contain exon 2 and exon 8 sequences can occur.

and sequencing, these bands were shown to correspond to the WT-PR mRNA, exon-6 deleted and exon-4 deleted PR variant mRNAs, respectively. Sequences of these variants showed a perfect junction between exons (31) surrounding the deletion area as shown in Figure 3. Two other bands, that migrated with the apparent size of 845 bp and 817 bp were also detected in some breast tumor samples, but at an apparent lower frequency than the bands corresponding to WT, exon 4-deleted and exon 6-deleted PR mRNAs. Sequencing of these bands identified a PR variant transcript containing a deletion of both exon 3 and 6, as well as a transcript containing a deletion of both exon 5 and 6 (data not shown). Some other PCR products, which were not reproducibly observed or whose size did not correspond to any putative exon-deleted PR variant mRNA have not yet been characterized. Differences between samples in PR wild-type and variant mRNAs signals are unlikely due to variable input of cDNA, since similar signals were obtained in all samples after amplification of the house-keeping GAPDH cDNA (data not shown). Generally, there was good agreement between ligand binding assay and the wild-type PR reverse transcription (RT) PCR product obtained. Specificity of the RT-PCR approach is demonstrated by the lack of signal in a PR negative tumor (T2), as measured by ligand binding assay. Table 1 summarizes the characteristics (size, predicted molecular mass, predicted isoelectric point) of the putative proteins encoded by the PR variants identified in this study. Because both PR-A and PR-B type variant mRNAs will be identified using our approach, both types of putative protein are presented (we have not analyzed our data with respect to the putative PR-C isoform). Intact functional domains that remain in the resulting protein are indicated for each PR-variant. It should be emphasized that to date, only RT-PCR bands corresponding to the exon 4-deleted and exon 6-deleted variant mRNAs in addition to the wild-type PR mRNA were reproducibly detected in normal breast tissue. Our data suggest that the exon 6-deleted transcript is more frequently detected in neoplastic breast tissues compared to the normal breast tissues examined in this study.

DISCUSSION

We have identified for the first time several exon-deleted PR variant mRNAs present in both normal and neoplastic breast tissues. As previously observed with exon-deleted ER

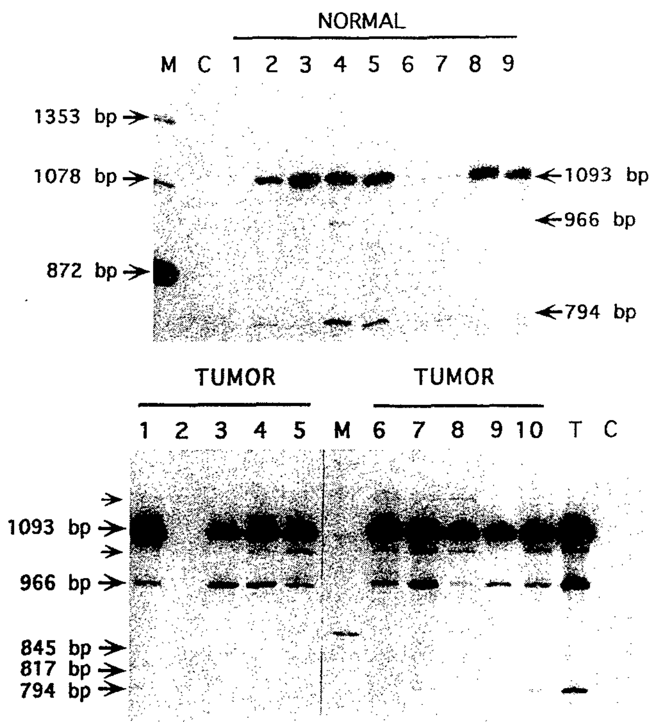

FIG. 2. Co-amplification of WT-ER and deleted variant mRNAs in human breast samples. Total RNA extracted from normal (Normal 1-9) and tumorous (Tumor 1-5 and 6-10) breast tissue samples or from T47D-5 breast cancer cells (T) was reverse transcribed and PCR amplified as described in the "Material and Methods" section using PRU2 and PRL2 primers. Radioactive PCR products were separated on a 4% acrylamide gel and visualized by autoradiography. Bands that migrated at 1093 bp, 966 bp, 845 bp, 817 bp and 794 bp were identified as corresponding to WT-PR mRNA and variant mRNAs deleted in exon 6, doubly deleted in exon 3-6, doubly deleted in exon 5-6 and deleted in exon 4, respectively. PCR products indicated by small arrows have not yet been characterized. M: Molecular weight marker (ϕx174 HaeIII digest, Gibco BRL, Grand Island, NY). C, no cDNA added during the PCR reaction.

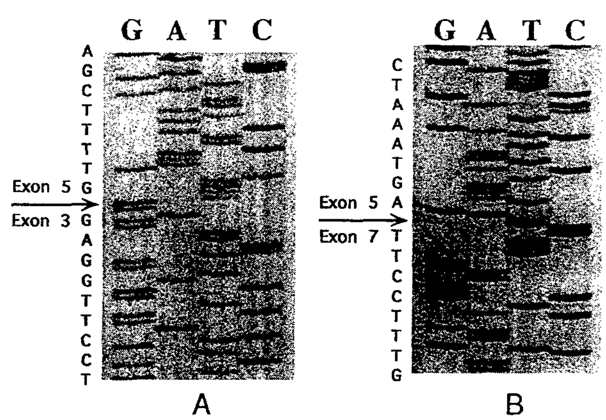

FIG. 3. PR-variant mRNAs sequences. PCR products were subcloned and sequenced as described. A: exon 4-deleted variant sequence, B: exon 6-deleted variant sequence.

TABLE 1

PR Variant mRNAs Identified within Human Breast Tissues and Putative Encoded Proteins

	PR-B mRNA	PR-A mRNA	Functional domains	Samples
Wild-type	933 aa	769 aa		
	Mr 99,035	*Mr* 82,350	A, B, C, D, E	N, T
	pI 6.07	pI 8.50		
Exon 4-deleted variant	831 aa	667 aa	A, B, E	
	Mr 87,788	*Mr* 71,103		N, T
	pI 5.62	pI 6.97	in frame	
Exon 6-deleted variant	797 aa	633 aa	A, B, C, D	
	Mr 83,017	*Mr* 66,332		N, T
	pI 5.55	pI 7.42	truncated, 12 new aa	
Exon 5-6-deleted variant	841 aa	677 aa	A, B, C, D	
	Mr 88,068	*Mr* 71,383		T
	pI 6.06	pI 8.71	in frame	
Exon 3-6-deleted variant	758 aa	594 aa	A, B, D	
	Mr 78,671	*Mr* 61,985		T
	pI 5.03	pI 5.80	truncated, 12 new aa	

Note. For each PR-A or PR-B variant mRNA, the size (in amino acids, aa) of the putative encoded protein, its predicted molecular weight (*Mr*, given in daltons) and its predicted isoelectric point (pI) are given. Functional domains that remain intact are indicated (A-E) as well as the amino acid composition change. Detection in normal (N) or in tumor (T) breast samples is also specified.

variant mRNAs, sequencing these variant mRNAs revealed a perfect junction between exons surrounding the deletion area. This suggests that these naturally occuring variants are generated by alternative splicing of WT-PR primary transcripts. Whether or not all groups of mRNAs (PR-A, PR-B, PR-C) are alternatively spliced remains to be determined. However, any of these variant species, if translated, would encode PR-like proteins which lack some functional domains of the WT-PR proteins. The resulting shorter proteins could contribute to the population of PR-related proteins observed in human breast tumor samples by Graham et al. (15), as suggested by these authors. For example, the putative protein encoded by the exon 4-deleted PR-A variant mRNA is expected to migrate at a apparent molecular mass of 71,103 daltons and could correspond to the PR-related protein (78,000 daltons) observed in 25.7% of the tumors analyzed by Graham et al. (15).

Several different sized PR mRNAs species have previously been described in human and chicken target tissues. Generation of these transcripts is thought to involve several mechanisms: different promoter usage, alternative polyadenylation site selection and a splicing variant have been identified (17, 18). The splicing variant, identified in chicken oviduct (18), seems to consist of a failure to splice the second intron followed by polyadenylation site selection within this intron. The resulting transcript therefore consists of exons 1 and 2 with some intron 2 sequences followed by a polyadenylation signal and a poly-A tail. However, no previous studies have identified complete exon-deletions in PR transcripts. Such deletions would have escaped detection by previous studies using Northern blot and differential hybridization analysis.

By analogy with ER variant mRNAs (20, 21, 32) it is reasonable to hypothesize that the putative encoded PR variant proteins, with structural and functional alterations, may modify WT-PR functions. The presence of PR variant mRNAs in normal tissue therefore suggests that the PR signalling pathway involves more protagonists than PR-A, PR-B or PR-C. Moreover, we showed that the detection of a particular variant using this kind of approach depended on the initial representation of this mRNA within the related-mRNAs population (30). Our results suggest that the differences in PR-variant mRNAs detection between samples may therefore

reflect different relative proportions of these variants within samples. Whether or not differential PR variant mRNA expression is associated with a pathophysiological role in progestin action is under investigation.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Breast Cancer Research Initiative (CBCRI) and the U.S. Army Medical Research and Material Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC) and the "Terry Fox Foundation." P. H. W. is a Medical Research Council of Canada (MRC) Clinician-Scientist, L. C. M. is an MRC Scientist. E. L. is a recipient of a USAMRMC Postdoctoral Fellowship Award.

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APPENDIX 9

EXPRESSION OF EXON 6 DELETED PROGESTERONE RECEPTOR VARIANT mRNA IN NORMAL HUMAN BREAST TISSUE AND BREAST TUMOURS[1]

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Running title: Exon 6-deleted PR variant in human breast tissue

Key words: Progesterone receptor, variant mRNAs, breast cancer, differential splicing, PCR.

Footnotes:

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[3] The abbreviations used are: PR, progesterone receptor; D6-PR, PR variant deleted in exon 6; ER, estrogen receptor; WT, wild-type; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.

Abstract

Expression of the recently described progesterone receptor (PR) variant mRNA deleted in exon 6 (D6-PR) was investigated by reverse transcription and polymerase chain reaction in ten normal reduction mammoplasties samples, nine breast tumors with high PR levels (>100 fmol/mg protein) and eight breast tumors with low PR levels (<15 fmol/mg protein), as determined by ligand binding assay. The relative expression of D6-PR to wild-type PR mRNA was lower (P<0.01) in normal than in tumor breast samples. Moreover, a trend to lower (P<0.1) relative D6-PR expression was observed in high PR tumors, compared to low PR tumors. This suggests that increased expression of D6-PR occurs during tumor progression.

Introduction

PR is an important prognostic marker in breast cancer (1). Estrogen receptor (ER) positive breast tumors that also contain PR (ER+/PR+ tumors) are considered as "good prognosis" tumors and are likely to respond to endocrine therapies (2, 3). In contrast, absence of PR often characterizes "poor prognosis" tumors (ER+ /PR-) and resistance to endocrine therapy (4).

Similar to other members of the steroid receptor superfamily, PR is divided into structural domains (A-E, Fig.1), the functions of which are widely documented (5). Two different PR isoforms, PR-A and PR-B, that are encoded by mRNAs transcribed from the same gene under the control of two different promoters (6) have been identified in both normal and neoplastic tissues (Fig.1). We recently described the presence of several exon-deleted PR variant mRNAs in both normal and neoplastic breast samples (7). Amongst them, a variant mRNA deleted in exon 6 (D6-PR mRNA) that if translated, would encode a PR-A- and/or a PR-B-like protein containing a truncated E domain. This variant would therefore be missing the hormone binding domain and one of the transactivating domains (TAF-2) of the wild-type (WT) PR protein. Because expression of similar exon-deleted or truncated variants have been associated with tumor progression in the case of ER variant mRNAs (8-14), it was of interest to determine whether D6-PR variant expression was also modified during tumorigenesis. In this study, we investigated D6-PR variant expression by reverse transcription and polymerase chain reaction (RT-PCR) in 10 normal reduction mammoplasties samples, 9

breast tumors with high PR levels (considered as "good prognostic" tumors), and eight breast tumors with low PR levels (considered as "poor prognosis" tumors).

Materials and methods

Human breast tissues

Human breast specimens (27 cases) were all collected at the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The presence of normal ducts and lobules as well as the absence of any atypical lesion were confirmed in all 10 normal reduction mammoplasties specimens. The seventeen primary invasive ductal breast carcinomas were associated with high ER levels ranging from 105 to 386 fmol/mg protein (mean = 190.7 fmol/mg protein, standard deviation = 58.49), as determined by ligand binding assay. Within this group, 9 tumors had a high progesterone receptor level (PR>100 fmol/mg protein, mean = 156.4 fmol/mg protein, standard deviation = 28.4) and 8 had a low progesterone receptor level (PR<15 fmol/mg protein, mean = 8.6 fmol/mg protein, standard deviation = 4.6), as determined by ligand binding assay. It should be stressed that among those later tumors, only one was PR negative (PR = 0). The ages of patients associated with the tumor samples ranged from 37 to 91 (mean: 70 years old, standard deviation: 14.4 years). For reduction mammoplasties, women were younger with ages ranging from 19 to 41 years old (mean: 31.3 years old, standard deviation: 8.3 years). Total RNA was extracted from frozen tissue sections and reverse transcribed in a final volume of 15 µl as previously described (14).

Polymerase chain reaction (PCR) and identification of PCR-products.

The primers used consisted of D6U primer (5'-CTCTCATTCAGTATTCTTGG-3'; sense; located in PR exon 5; 2989-23008) and D6L primer (5'-TGGGTTTGACTTCGTAGC-3'; antisense; located in PR exon 7; 3262-3245). The nucleotide positions correspond to published sequences of the human PR cDNA (6). PCR amplifications were performed and PCR products analyzed as previously described with minor modifications (14). Briefly, 1 µl of reverse transcription mixture was amplified in a final volume of 10 µl, in the presence of 10 nM [α-^{32}P] dCTP, 4 ng/µl of each primer and 1 unit of Taq DNA polymerase. Each PCR consisted of 40 cycles (30 s at 60°C and 30 s at 94°C). As positive controls, aliquots of plasmid DNA containing previously (7) subcloned WT-PR (WT cont) or exon 6-deleted PR (D6 cont) sequences were amplified in

parallel. PCR products were then separated on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and autoradiographed. The PCR product corresponding in size to D6-PR was subcloned and sequenced as previously described using D6U and D6L primers (14).

Quantification and statistical analysis

The approach used to evaluate the exon-deleted variant mRNA expression relative to WT mRNA has already been described for exon-deleted ER variant mRNAs (14, 15). PCR co-amplification of WT and exon-deleted variant generates 2 bands whose ratio is constant with varying cycle number and is independent of initial input cDNA. This assay provides a semi-quantitative RT-PCR whose internal control is the WT mRNA co-amplified and in which relative expression of variant mRNA can be determined for individual samples. Bands corresponding to D6-PR and WT mRNAs were excised from the gel and corresponding signals were subsequently measured after addition of 5 ml scintillant (ICN Pharmaceuticals, Inc., Irvine, CA) by counting in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The D6-PR signal was expressed as a percentage of the WT-PR signal. For each sample, 3 independent assays were performed and the mean determined. The statistical significance of differences in the relative levels of expression of D6-PR mRNAs was determined using the Mann-Whitney rank-sum test (two-sided).

Results

Detection of D6-PR in all normal and tumor breast tissues.

Total RNA from ten normal breast tissues and seventeen breast tumor specimens was analyzed by RT-PCR as described in "Material and Methods" section using primers depicted in Figure 1. These primers were designed to allow the co-amplification of D6-PR and WT-PR mRNAs. Among the seventeen tumors studied, 9 had a high PR level (>100 fmol/mg protein) and 8 a low PR level (<15 fmol/mg protein), as determined by ligand binding assay. In each sample, two bands that corresponded in size to that expected for WT-PR and D6-PR PCR products were obtained. Figure 2 presents a typical autoradiograph after 1 night exposure. It should be noted that a longer exposure or addition of intensifying screens allowed the detection of D6-PR in both lanes N3 and LPR3 (data not shown). The PCR product corresponding in size to D6-PR was subcloned

and subsequently sequenced. Sequence analysis showed the expected perfect junction between exon 5 and exon 7 (data not shown).

Comparison of D6-PR variant expression in normal and tumor tissues

The D6-PR variant mRNA expression relative to WT-PR was then evaluated in each sample. It has been previously demonstrated that the co-amplification of WT and exon-deleted variant transcripts led to the synthesis of two PCR products. Further, the ratio of the signals obtained from these two products ratio could be used to compare relative exon-deleted variant expression within samples (14, 15). Bands corresponding to WT-PR and D6-PR were therefore excised from the gels and corresponding signals subsequently quantified as described in "Material and Methods" section. The signal corresponding to D6-PR was expressed as a percentage of the WT-PR signal and the mean of three different assays calculated (Fig.3). The level of exon 6-deleted variant mRNA relative to the WT-PR mRNA was found to be significantly ($P<0.05$) lower in normal (median = 4.8%) than in neoplastic breast tissues having either high PR or low PR (median = 9.19% and median = 25.13%, respectively). The significance became higher ($P<0.01$) when the tumors were considered together (median = 13.86%). Moreover, even though the difference did not achieve statistical significance ($0.1<P<0.05$), D6-PR relative expression appeared lower in tumors with high PR levels (median = 9.19%) than in tumors with low PR levels (median = 25.13%).

Discussion

We have previously detected a PR variant mRNA deleted in exon 6 in several, but not all, normal and tumor breast samples (7). Our previous study suggested that the relative level of this PR variant mRNA was higher in tumor versus normal breast tissues. However, the approach used in this initial study was designed to qualitatively assess deletion and insertion PR variant expression and its quantitative rigor has not been assessed. The specificity of the approach used here, i.e specific amplification of the studied PR mRNA region (between exon 5 and exon 7) versus general amplification of all exon-deleted PR variant mRNAs (between exon 2 and 8) used in our first study, has been validated, previously, and is probably the reason for the more general detection of the exon 6-deleted PR transcript. The perfect junction of exon 5 and exon 7 that characterizes D6-PR together with its detection in all samples, strongly suggests that this naturally occuring

variant is generated by alternative splicing of WT-PR primary transcripts. Whether or not all groups of primary transcripts previously identified as WT-PR (i.e PR-A and PR-B mRNAs) are involved in the alternative splicing events remains an open question. Whatever the answer to this question, D6-PR mRNA, if translated, would encode a PR-A and/or a PR-B-like protein lacking the hormone binding domain of the WT molecule (7). Depending on the phosphorylation state of the protein and other factors that may influence mobility on denaturing gel electrophoresis, it is possible that the resulting shorter proteins may not be distinguishable from the WT-PR-A isoform and may contribute to PR-A signals obtained on 1D PAGE. No data are available as yet concerning the function of such putative D6-PR proteins. We can therefore only speculate on such possible functions by analogy with what has previously been shown for other steroid receptor proteins truncated in the hormone binding domain. For example, an artificial PR variant, truncated at the carboxy (C)-terminal 42 amino acid containing a part of the hormone binding domain, had lost the ability to bind either progesterone or the progestin R5020 but could still bind the antiprogestin RU486 but now activated transcription in its presence (16). This suggests that the extreme C-terminal region of PR contains an inhibitory function that silences receptor transactivation in the absence of agonist and in the presence of antagonist. The absence of this region, also observed in D6-PR protein, may confer similar properties to this variant PR. Similarly, naturally occuring ER variants lacking the hormone binding domain of the WT molecule have been shown to be able to interfere with WT-ER signalling pathway (8-10, 17). For example, exon 7-deleted ER variant was shown to act as dominant negative regulator of WT ER (9, 17) whereas exon 5-deleted ER has ligand independent trans-activating activity (8). Similar modifications of the WT-PR signalling pathways due to D6-PR variant could also be suggested.

Interestingly, relative levels of some of the ER variants cited above were found to be increased during tumor progression. Exon 7-deleted variant mRNA level was shown to be higher in ER+/PR- versus ER+/PR+ tumors (9). Exon 5-deleted ER variant mRNA expression was found higher in ER-/PR+ versus ER+/PR+ tumors (8, 11) and was decreased in normal versus tumor breast tissues (14). It has thus been speculated that these ER variants may be involved in progression from hormone dependence to independence in breast cancer (18, 19). This aspect of tumor progression consists in the acquisition of resistance to the cytostatic effects of the antiestrogen tamoxifen and subsequently in the failure to respond to agents such progestins

and probably antiprogestins (RU 486) (20, 21). The apparent lower relative expression of D6-PR in normal breast samples compared to tumor tissues as well as in high PR tumors compared to low PR tumors is therefore of interest, since normal tissue, high PR tumors and low PR tumors parallel a progression series, which correlates with increased relative D6-PR expression. Because this transcript could encode a truncated PR protein with altered function, a role for PR variant proteins in breast tumor progression and progestin/antiprogestin resistance could be evoked. Moreover, because the two tumor subsets studied here belong to two subgroups previously shown to differ in terms of prognosis and disease free survival (22), one can therefore speculate than D6-PR relative expression may correlate with various steps of tumor progression and provide a new prognostic marker. In order to clarify such issues, larger numbers of samples require screening for D6-PR expression. Further characterization of the putative functions of D6-PR-A and D6-PR-B proteins in the presence of progestin and/or antiprogestin ligands requires investigation in laboratory models of normal and neoplastic breast tissues.

The measurement of PR is an important tool in clinical decision making with respect to prognosis and treatment of human breast cancer. Furthermore, the level of PR expression provides important clinical information as shown by Clark et al. (22). As the use of enzyme-linked immunosorbent assays (ELISA) and immunohistochemical assays for PR detection increases, it is likely that variant PR expression will interfere with these assays. Anti- human PR antibodies (as AB-52 antibody, (23)) used in such assays detect epitopes in the N-terminus extremity of the WT molecule shared by truncated PR-like molecules. If D6-PR variant mRNAs are translated into stable proteins, they will be co-detected with the WT-PR in an assay using such an antibody (24). Presence of PR variants such as D6-PR may also be considered when discrepancies between biochemical measurement and immunological detection of PR are observed (25).

In conclusion, we show in this pilot study that exon 6-deleted PR variant mRNA relative expression is increased during breast tumor progression. It is our speculation that PR variants may have a role in tumorigenesis, and/or be a marker of breast cancer progression, as already suggested for ER variants.

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Figures legend

Figure 1

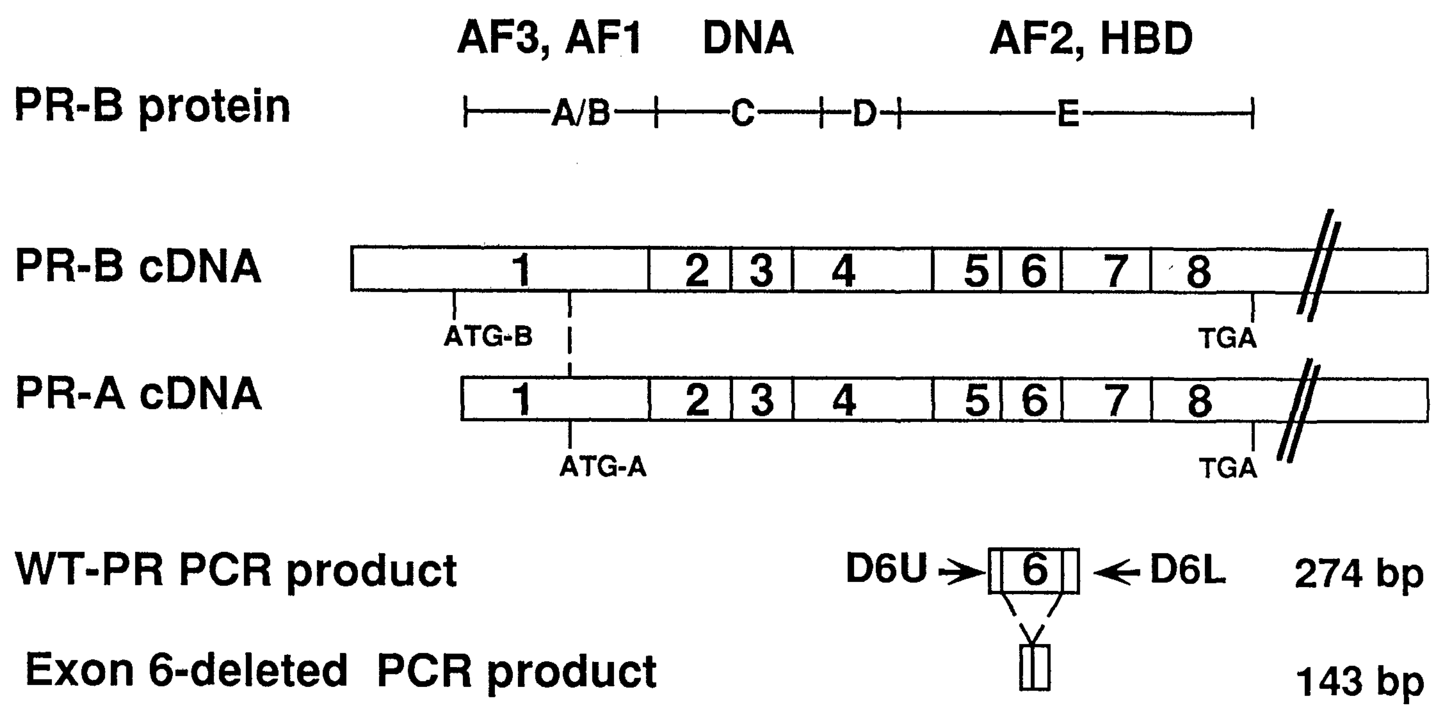
Schematic representation of PR-B protein, PR-B and PR-A cDNAs and primers used to co-amplify WT-PR and D6-PR variant cDNAs. PR cDNAs contain 8 different exons coding for a protein divided into structural and functional domains (A-E). A/B region of the receptor contains two transactivating domains (AF3 and AF1). The C region contains the DNA binding domain whereas region E, that is involved in hormone binding, contains another transactivating domain (AF2). ATG-B and ATG-A are the translational start sites of PR-B and PR-A proteins, respectively. TGA, stop codon. D6U and D6L primers allow co-amplification of a 274 bp and 143 bp fragments corresponding to WT-PR and D6-PR mRNAs, respectively.

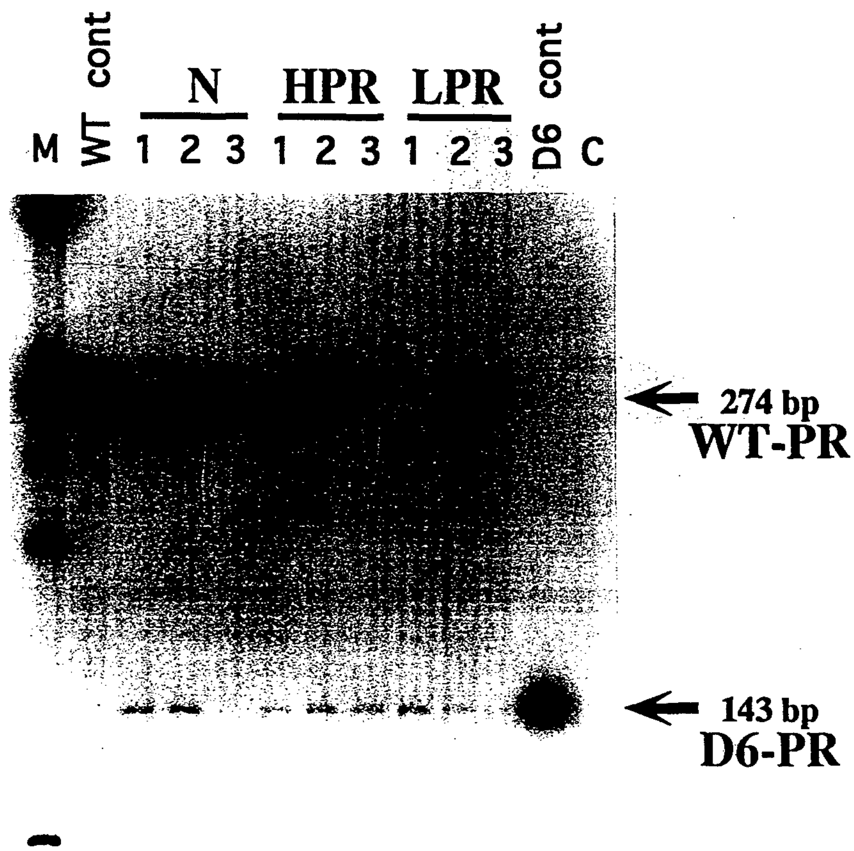
Figure 2

Detection of exon 6-deleted PR variant mRNA in all human breast samples. Total RNA extracted from normal (N1-3), high PR tumor (HPR1-3) and low PR tumor (LPR1-3) breast tissue samples was reverse transcribed and PCR amplified as described in the "Material and Methods" section using D6U and D6L primers. Radioactive PCR products were separated on a 6% acrylamide gel and visualized by autoradiography. Bands that migrated at 274 bp and 143 bp were identified as corresponding to WT-PR and exon 6-deleted PR variant mRNAs, respectively. Plasmids containing either WT-PR (WT cont) or exon 6-deleted PR (D6 cont) sequences were used as positive control. M: Molecular weight marker (ϕx174 HaeIII digest, Gibco BRL, Grand Island, NY). C, no cDNA added during the PCR reaction.

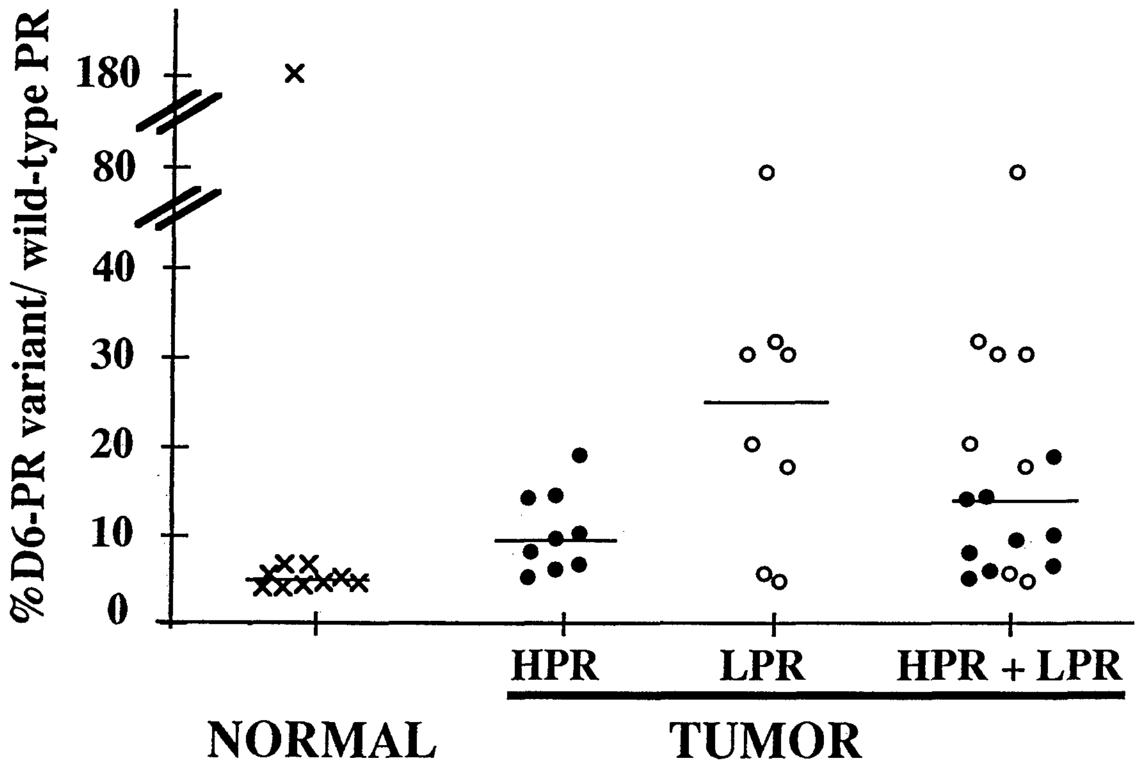
Figure 3

Comparison of D6-PR relative expression between normal and tumor samples. Total RNA extracted from 10 normal breast samples (x), 9 tumors with high PR (black circles, HPR) and 8 tumors with low PR (white circles, LPR) was reverse transcribed and PCR amplified as described in the "Material and Methods" section using D6U and D6L primers. D6-PR corresponding signal was measured as described in the "Material and Methods" and expressed as a percentage of wild-type PR corresponding signal. For each sample, the mean of three different experiments is indicated. Bars: medians.


AF3, AF1
DNA
AF2, HBD
PR-B protein
A/B
C
D
E
PR-B cDNA
1
2
3
4
5
6
7
8
ATG-B
TGA
PR-A cDNA
ATG-A
WT-PR PCR product
D6U
D6L
274 bp
Exon 6-deleted PCR product
143 bp



M
WT cont
N
HPR
LPR
1 2 3 1 2 3 1 2 3
D6 cont
C
274 bp
WT-PR
143 bp
D6-PR



%D6-PR variant/ wild-type PR
180
80
40
30
20
10
0
HPR
LPR
HPR + LPR
NORMAL
TUMOR

APPENDIX 10

Journal of Cellular Biochemistry 63:174–184 (1996)

Estrogen Regulation of Nuclear Matrix-Intermediate Filament Proteins in Human Breast Cancer Cells

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Abstract The tissue matrix consists of linkages and interactions of the nuclear matrix, cytoskeleton, and extracellular matrix. This system is a dynamic structural component of the cell that organizes and processes structural and functional information to maintain and coordinate cell function and gene expression. We have studied estrogen regulation of nuclear matrix associated proteins, including the intimately connected cytoskeletal intermediate filaments, in T-47D5 human breast cancer cells. Three proteins (identified as cytokeratins 8, 18, and 19) present in the nuclear matrix-intermediate filament fraction (NM-IF) of cells grown in estrogen-replete conditions were dramatically reduced when the cells were grown in acute (1 week) estrogen-depleted conditions. Replacing estrogen in the medium of acute estrogen-depleted cells restored expression of these proteins. T-47D5 cells that are chronically depleted of estrogen (T5-PRF) are estrogen-nonresponsive in culture. These cells overexpressed these three proteins, compared to parent cells grown in the presence of estrogen. Treatment of the T5-PRF cells with estrogen did not lead to further up-regulation of these proteins. Treating T-47D5 cells in estrogen-replete conditions with the antiestrogens 4-hydroxytamoxifen and ICI 164 384 (100 nM, 3 days) resulted in a significant reduction in these proteins, while no effect was seen in long-term chronic estrogen-depleted T-47D5 cells. In conclusion, we have identified NM-IF proteins (cytokeratins 8, 18, and 19) in human breast cancer cells that are estrogen regulated and may play a role in estrogen action in human breast cancer cells. © 1996 Wiley-Liss, Inc.

Key words: cytokeratins, hormone independence, T-47D5, nuclear matrix, breast cancer

The tissue matrix system, consisting of dynamic linkages between the nuclear matrix (NM), the cytoskeleton, and the extracellular matrix (ECM) forms a structural and functional connection from the cell periphery to the DNA [Pienta and Coffey, 1992]. The cytoskeleton is composed, in part, of intermediate filaments (IFs). Cytokeratins are members of the intermediate filament family of structural proteins [Moll et al., 1982]. Direct connections (via intermediate filaments) between the cell periphery and NM have been demonstrated and this nuclear matrix-intermediate filament (NM-IF) system is altered by tumour promoters [Fey and Penman, 1981; Fey et al., 1984]. Direct evidence of a continuous network connecting the plasma membrane structure and cytoskeleton with the nucleoskeleton of eukaryotic cells is provided by data demonstrating that vimentin is anchored directly to the nuclear lamina via lamin B [Djabali et al., 1991; Georgatos and Blobel, 1987]. Evidence also suggests that the intermediate filaments, in particular the lamins, not only exist at the nuclear periphery but are also found as part of the internal nuclear matrix [Hozak et al., 1995; Martell et al., 1992].

Architectural alterations (defined by structural NM proteins and/or interactions with the cytoskeleton) within or associated with the nucleus may influence or control what genes or subsets of genes are actively transcribed. Nucleic acids can interact with IFs [Traub et al., 1983] and, disrupting the cytoskeleton with cytochalasin D (an actin microfilament inhibitor), induce specific gene expression [Zambetti et al., 1991]. Manipulation of the cytoskeleton can alter the pattern of gene expression [Blum and Wicha, 1988] and in mammary epithelial cells, the ECM has been shown to regulate tissue specific gene expression [Boudreau et al., 1995; Streuli et al., 1995]. Seely and Aggeler [1991] have demonstrated that modulating the cytoskeleton in cultured mouse mammary epithelial cells altered

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milk protein synthesis. Together these data demonstrate that not only is the cytoskeleton physically connected to, and may even be considered, an intimate part of the nucleus/NM structure, but that the nucleus can respond to signals from the structural organization of the cytoskeleton in order to modulate gene expression. Because of the intimate connection of the NM with the cytoskeleton, our study is concerned with examining the effects of hormone on NM proteins and IF proteins associated with the nuclei in human breast cancer cells.

The nuclear matrix plays an important role in many nuclear processes, including DNA organization [Getzenberg et al., 1991], replication [Cook, 1991], gene transcription and processing [Getzenberg and Coffey, 1991; Huang and Spector, 1991; Xing et al., 1993; Carter et al., 1993], and steroid hormone action [Kirsh et al., 1986; Alexander et al., 1987]. The expression of several NM proteins is cell [Fey and Penman, 1989], tissue [Getzenberg and Coffey, 1990], differentiation [Dworetsky et al., 1990], and hormonal state specific [Getzenberg and Coffey, 1990]. Differences in NM protein expression exist between normal tissues and their cancerous counterparts [Partin et al., 1993], and more specifically, differences have been shown between normal breast tissue and breast cancer [Khanuja et al., 1993]. Steroid hormone receptor complexes interact with the nucleus and subsequently modulate gene expression, but a comprehensive understanding of the mechanisms involved is lacking. Several steroid hormone receptors, including the estrogen receptor, have been shown to localize to the NM. Cell-free binding assays confirm that this localization is due to the presence of specific acceptor sites in the NM to which steroid–receptor complexes bind with high affinity and tissue specificity [Metzger and Korach, 1990]. Patterns of NM protein expression are hypothesized to be involved in changes in gene expression and it is believed that the specific proteins of the NM can influence gene expression [Stein et al., 1994; Bidwell et al., 1993]. The way in which the protein changes in the NM could influence gene expression is unclear, but transcriptionally active genes have been shown to be associated with the nuclear matrix, whereas inactive genes are not [Getzenberg et al., 1991; Gerdes et al., 1994].

Breast cancer is a hormonally responsive cancer and is dependent on estrogen for growth [Dickson and Lippman, 1991]. Estrogens promote the growth of human breast cancer and, as such, many therapies are aimed at blocking the growth promoting effects of estrogen (e.g., antiestrogens). The evolution of breast cancer into an estrogen-independent growth phenotype marks the beginning of a more aggressive phase of the disease and is a major problem in the efficacy of antiestrogen treatments [Clarke et al., 1990; Leonessa et al., 1991]. Understanding the factors that contribute to the development of a hormone-independent phenotype is of major importance in terms of breast cancer therapeutics.

Several breast cancer cell lines in culture also require estrogen for growth and long-term culture in estrogen-depleted conditions can result in these cells becoming independent of the requirement for estrogen for growth. Indeed, the development of estrogen-nonresponsive growth in human breast cancer is thought to be one of the initial steps in the progression to hormone independence [Clarke et al., 1994].

This study is concerned with examining the effects of estrogen on NM-IF expression in estrogen-responsive (ER+) and estrogen-nonresponsive (ER+) human breast cancer cells. We identified three NM-IF proteins—cytokeratin 8 (CK8), 18 (CK18), and 19 (CK19)—that are estrogen-regulated in T-47D5 human breast cancer cells. In T-47D5 cells that are estrogen independent in culture, these cytokeratins are overexpressed and are no longer sensitive to regulation by either estrogen or antiestrogens.

MATERIALS AND METHODS

Materials

Dulbecco's minimal essential medium (DMEM) and phenol red free DMEM powder were purchased from GIBCO/BRL (Burlington, Ontario). Fetal bovine serum (FBS) was purchased from UBI (Lake Placid, NY) and all other cell culture ingredients were purchased from Flow Laboratories (Mississauga, Ontario). 4-Hydroxytamoxifen and ICI 164 384 were gifts from ICI (Macclesfield, Cheshire) and estradiol was purchased from Sigma. ^{3}H-Estradiol and ^{3}H-R5020 were purchased from NEN (Mississauga, Ontario).

Cells and Cell Culture

T-47D5 [Watts et al., 1992], T-47D, MDA MB 231, and HBL 100 human breast epithelial cells

were routinely grown in DMEM supplemented with 5% FBS, glucose, glutamine, and penicillin–streptomycin (5% CM) as previously described [Shiu, 1979]. T-47D5 chronically estrogen-depleted cells (T5-PRF) were routinely grown in phenol red-free DMEM supplemented with 5% 2× charcoal-stripped fetal calf serum (CS-FCS), glucose, glutamine, and penicillin–streptomycin (5% CS). T-47D5 cells acutely depleted of estrogen were grown in 5% CS medium for one passage. Cells were passaged at 70–80% confluency using Earle's EDTA solution. Cells for nuclear matrix isolation were harvested at ~80% confluency using a rubber policeman, pelleted by centrifugation and stored at −70°C until processing.

Development of T-47D5 Chronically Estrogen-Depleted Cell Line

T-47D5 parent cells were passaged into phenol red-free DMEM supplemented with 5% 2× charcoal-stripped CS-FCS, penicillin–streptomycin, glucose, and glutamine (5% CS). Cells were routinely passaged at 70–80% confluency using Earle's EDTA solution. Long-term chronically estrogen-depleted T-47D5 cells have been depleted of estrogen for at least 60 passages, while the short-term estrogen-depleted cells have been grown in estrogen-depleted conditions for at least 10 passages. Acutely estrogen-depleted T-47D5 cells are passaged once in 5% CS.

Growth Experiments

Growth experiments were routinely performed by setting up cells at 10^4 cells/35-mm dish [Murphy et al., 1990]. All growth experiments on chronically estrogen-depleted T-47D5 cells were performed in 5% CS. Estrogen growth experiments on T-47D5 cells were performed in 5% CS, while antiestrogen growth experiments were performed in 5% CM. Two days later, fresh medium was added, which contained the appropriate concentration of drug to be tested from 1,000× stock solutions in ethanol. After 5 days the cells were harvested in triplicate using trypsin/EDTA and counted using an electronic cell counter (Coulter Electronics, Burlington, Ontario). Results were expressed as proliferation rate (percentage control) using the equation

$$\text{Doubling time (DT)} = 2\,\text{logn}/\log\,(\text{Tn}/\text{Ti})$$

where Ti is initial cell number, Tn is final cell number and n is the time (days) between T_i and T_n. Proliferation rate as a percentage of control was then calculated from the

$$\text{Proliferation rate} = \text{doubling time (control)} \times 100/\text{doubling time}$$

Isolation of NM-IF

Nuclei were extracted with TNM buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris–HCl pH 7.4, 2 mM $MgCl_2$, 1% (v/v) thiodiglycol) containing 1 mM PMSF and 0.5% (v/v) Triton X-100, and nuclear matrices were prepared essentially as previously described [Sun et al., 1994]. Briefly, nuclei (20 A_{260}/ml) were resuspended in digestion buffer (50 mM NaCl, 300 mM sucrose, 10 mM Tris–HCl pH 7.4, 3 mM $MgCl_2$, 1% (v/v) thiodiglycol, 0.5% (v/v) Triton X-100) and digested with DNase I (168 U/ml) for 20 min at room temperature. Ammonium sulfate was added dropwise from a 4 M stock, to a final concentration of 0.25 M, and the nuclear matrix was pelleted by centrifugation. The ammonium sulfate-extracted nuclear matrix was resuspended in digestion buffer and re-extracted by slowly adding NaCl to a final concentration of 2.0 M from a 4.0 M stock solution with mixing. This was left on ice for 30 min and then pelleted by centrifugation. The nuclear matrix was again re-extracted with 2 M NaCl and 1% (v/v) 2-mercaptoethanol for 30 min on ice and the insoluble nuclear matrix isolated by centrifugation. This fraction (NM-IF) contains nuclear matrix proteins and associated intermediate filaments [Fey et al., 1984]. Nuclear matrices were resuspended in 8 M urea and stored at −20°C. Protein levels were assayed using BioRad (Bradford) protein assay kit (Mississauga, Ontario).

Steroid Hormone Receptor Assay

Whole cell binding assays for estrogen receptors were performed as previously described [Murphy and Dotzlaw, 1989].

SDS–PAGE, Coomassie Staining, and Quantitation

Subcellular fractions were analyzed under reducing conditions by electrophoresis on 12% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) with 4% stacking gel at 200 V for 45 min at room temperature according to the Laemmli method [Laemmli, 1970]. Gels were stained in 0.05% Coomassie Blue R-250. Quantitation of cytokeratin levels was performed on

Coomassie Blue stained gels using scanning densitometry and lamin bands (identified using immune detection along with purified lamins as standards, gifts from Dr. Y. Raymond) were used as loading controls.

2-D Gel Electrophoresis

First-dimension isoelectric focusing was performed according to the method of O'Farrell [O'Farrell, 1975] and the second-dimension (2-D) SDS–PAGE according to the method of Doucet [Doucet and Trifaro, 1988]. Gels were stained using the silver staining technique [Heukeshoven and Dernick, 1985].

Protein Purification

NM-IF samples were prepared as described and resuspended in 7 M urea, 20 mM Tris–HCl, pH 8. Samples were ran over 1 ml Poros PI (anion-exchange) column at a protein concentration of 8 mg/column. Proteins were eluted with a linear gradient of 0–0.5 M NaCl in 15 ml and 0.6 ml collected/fraction. Fractions were assayed by SDS–PAGE and samples containing protein bands of interest were pooled. 2-D gels were run on pooled samples (100 μg/gel), gels were coomassie stained and spots excised. Excised spots were sent to the WM Keck Foundation (New Haven, CT) for internal microsequencing.

Western Blotting and Immune Detection

NM-IF samples were run on 12% SDS–PAGE with 4% stacking gel at 200 V for 45 min at room temperature according to the Laemmli method [Laemmli, 1970]. Gels were transferred to nitrocellulose as previously described [Delcuve and Davie, 1992] and transferred for 1½ hr at 120 V. Blots were blocked overnight at 4°C in 5% skim milk/Tris-buffered saline. Blots were incubated with 1° and 2° antibodies for 1 hr at room temperature in 2% skim milk/Tris-buffered saline containing 0.2% Tween-20. Detection was carried out using the ECL detection system according to the manufacturer's instructions (Amersham, Buckinghamshire, England).

RESULTS

Development of Hormone-Nonresponsive T-47D5 Subline

T-47D5 human breast cancer cells are estrogen receptor positive (ER^+) and estrogen treatment in culture results in increased proliferation of these cells (Fig. 1A). An estrogen-nonresponsive cell line was developed from this parent line, by chronically depleting the cells of estrogen in long-term culture (~60 passages). These cells are insensitive to the growth-stimulatory effects of estrogen seen in the parent cell line (Fig. 1A). Both the parent T-47D5 cells and the estrogen-nonresponsive (T5-PRF) cell line express the estrogen receptor, localized to the NM, as assayed by immune-detection (data not shown) and ligand-binding techniques (Table I). The T5-PRF cell line contained significant amounts of ER, although the absolute level of expression is reduced by ~50%, as compared to the parent line.

Antiestrogens antagonize the growth-stimulatory effects of estrogens and are often used in the treatment of human breast cancer [Jordan, 1988]. The antiestrogens ICI 164 384 and 4-monohydroxytamoxifen both result in the inhibition of T-47D5 cell growth (Fig. 1B,C). T-47D5 cells chronically grown in estrogen-depleted conditions still retain sensitivity to the growth inhibitory effects of 4-monohydroxytamoxifen and to a lesser extent to those of ICI 164 384 (Fig. 1B,C).

Total NM-IF Proteins in T-47D5 Cells

The T5-PRF cell line, while still retaining ER expression, is estrogen-nonresponsive, providing a model in which to examine the NM-IF profile between hormone-responsive and -nonresponsive human breast cancer cell lines. Total NM-IF protein expression was examined in T-47D5 human breast cancer cells. Subcellular fractions obtained during the NM-IF isolation procedure were analyzed by SDS–12% PAGE. Initially, the method for NM-IF preparation stopped at 0.25 M ammonium sulphate. However, the NM-IF fraction still contained significant amounts of histone contamination (Fig. 2, lane 4). This contamination was markedly decreased by sequential salt extractions of the NM-IF preparations as shown. The final protein composition of NM-IF isolated by sequential salt extractions of nuclei is shown in Figure 2 (lane 7). Arrows denote 41-, 45-, and 54-kD protein bands, which are enriched in the high-salt extracted NM-IF fraction.

Estrogen Regulation of NM-IF Proteins

NM-IF composition was compared between the parent T-47D5 cells and T5-PRF human breast cancer cell lines. The NM-IF of cells

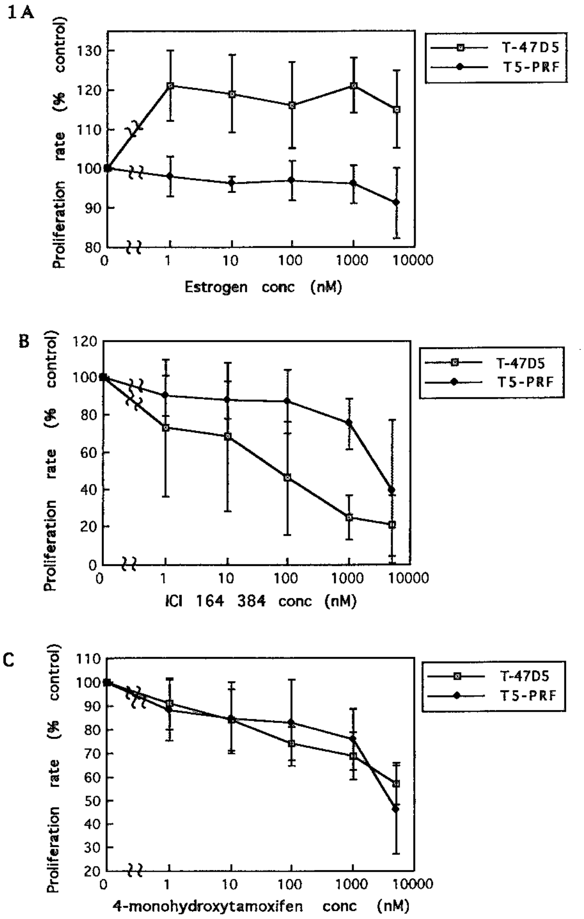


Fig. 1. Effect of estrogen and antiestrogen on proliferation of T-47D5 human breast cancer cells grown routinely in estrogen-replete (T-47D5) and estrogen-deplete (T5-PRF) conditions. The effects of increasing concentrations of estrogen (**A**) and antiestrogen (ICI 164 384 (**B**) and 4-monohydroxytamoxifen (**C**) on proliferation rate (percentage control). Approximately 10^4 cells were plated in duplicate on day −2. On day 0, three dishes were counted and cells were treated with complete medium containing estradiol or the appropriate antiestrogen to a final concentration of 0, 1, 10, 100, 1,000, and 5,000 nM. Five days later, cells were harvested and results expressed as proliferation rate as a percentage of control (see under Methods for formulae). Results represent the mean ±SEM, n = 3.

TABLE 1. Estrogen Receptor Levels in T-47D5 and T5-PRF Breast Cancer Cells.*

Cell line	ER sites/cell (±SEM)
T-47D5	186010 ± 37659
T5-PRF	71922 ± 29001

*Results are expressed as estrogen receptor sites/cell ±SEM, n = 5.

chronically depleted of estrogen (T5-PRF) contained elevated levels of the 41-, 45-, and 54-kD proteins compared to the parent, T-47D5, cell line grown in 5% CM (Fig. 3, lanes 1 and 3). This

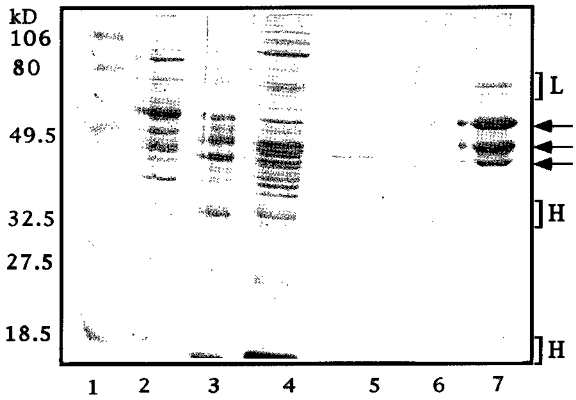


Fig. 2. NM-IF protein profile in T-47D5 human breast cancer cells. T-47D5 human breast cancer cells were grown in the presence of 10 nM estradiol for 72 h. NM-IF was isolated as described under Methods. *Lane 1,* MW standards (BioRad); *lane 2,* cytosol; *lane 3,* whole nuclei; *lane 4,* S1 = 0.25 M $(NH_4)_2SO_4$ NM-IF pellet; *lane 5,* S2 = 2 M NaCl solubilized fraction; *lane 6,* S3 = 2 M NaCl/1% 2-mercaptoethanol solubilized fraction; *lane 7,* NM-IF pellet. Subcellular fractions were run on 12% SDS–PAGE and gels were stained with Coomassie Blue to visualize protein bands. *Arrows,* 54-, 45-, and 41-kD protein bands. H, histones (identified based on relative mobility on SDS–PAGE against purified histones); L, lamins (identified using immune detection along with purified lamins as standards).

apparent overexpression appeared only in long-term chronic estrogen-depleted cells, since acute (one passage) estrogen withdrawal results in a marked reduction of these protein bands. T-47D5 cells passaged in estrogen-deplete conditions for ~10 passages, have levels comparable to those seen in the parent cell line (Fig. 3, lanes 5 and 7). The data suggested that the 41-, 45-, and 54-kD proteins may be estrogen regulated. This was confirmed by replacing estrogen in the medium of acutely estrogen-depleted cells, which resulted in increased expression of the 41-, 45-, and 54-kD proteins (Fig. 3, lanes 5 and 6). The expression of these three NM-IF proteins can also be further up-regulated by estrogen in T-47D5 cells and short-term chronic estrogen-depleted cells but exhibit little or no sensitivity to estrogen in the long-term chronic estrogen depleted T5-PRF cells (Fig. 3).

The effect of estrogen on these proteins was examined more closely. The results of a dose-response experiment, 72 h after estrogen treatment, are shown in Figure 4. Dose-response analyses show that as little as 0.1 nM estrogen resulted in increased levels of these proteins in the NM-IF of acute estrogen-depleted T-47D5 cells. Comparing the abundance of these proteins to the lamins (Fig. 4), it can be seen that the lamins are the most abundant NM-IF pro-

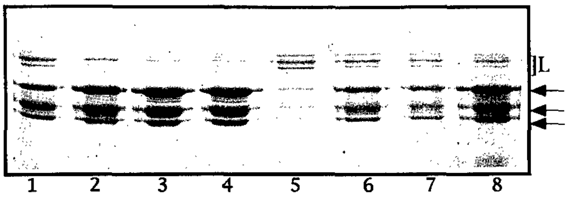
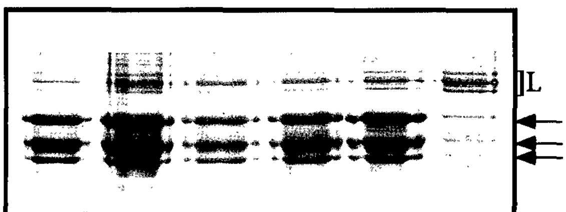


Fig. 3. NM-IF in estrogen-replete and estrogen-deplete conditions. NM-IF was obtained as described under Methods. *Lane 1,* T-47D5 NM-IF (ethanol vehicle); *lane 2,* T-47D5 NM-IF (10 nM estrogen (E2), 72 h); *lane 3,* T5-PRF NM-IF (ethanol vehicle); *lane 4,* T5-PRF NM-IF (10 nM E2, 72 h); *lane 5,* acute estrogen-deplete T-47D5 NM-IF (ethanol vehicle); *lane 6,* acute estrogen-depleted T-47D5 NM-IF (10 nM E2, 72 h); *lane 7,* short-term estrogen-depleted T-47D5 NM-IF (10 passages, ethanol vehicle); *lane 8,* short-term estrogen-depleted T-47D5 NM-IF (10 nM E2, 72 h). *Arrows,* 54-, 45-, and 41-kD protein bands; 5 μg protein/lane. L, lamins.



1	2	3	4	5	6
283+/-134	320+/-133	261+/-82	263+/-109	276+/-115	100

Fig. 4. Estrogen dose-response effects on NM-IF protein levels in acute estrogen-depleted T-47D5 cells. Cells were treated with estradiol at the appropriate concentration or vehicle control for 72 h. Dishes were harvested and NM-IF isolated as described under Methods. *Lane 1,* 1 μM E2; *lane 2,* 100 nM E2; *lane 3,* 10 nM E2; *lane 4,* 1 nM E2; *lane 5,* 0.1 nM E2; *lane 6,* ethanol vehicle. *Arrows,* 54-, 45-, and 41-kD protein bands; 5 μg protein/lane. L, lamins. Numbers below represent cytokeratin levels as a percentage of control ±SEM. Lamin bands were used as a loading control; n = 4.

teins in lane 6, but after estrogen treatment the 41-, 45-, and 54-kD protein bands are much more abundant.

Effect of Antiestrogen on NM-IF Proteins

The effects of the antiestrogens on the expression of the 41-, 45-, and 54-kD proteins were next studied. Treating T-47D5 cells, grown in the presence of estrogen, for 72 h with either 100 nM ICI 164 384 or 4-monohydroxytamoxifen (OT) resulted in a significant reduction in the NM-IF levels of these proteins (Fig. 5A). The amounts of these three proteins associated with the NM-IF fraction of long-term chronically estrogen-depleted T-47D5 cells (T5-PRF) did not appear to be affected by antiestrogen treatment (Fig. 5B). Thus, although antiestrogens inhibit the growth of these cells, the inhibitory effect on the abundance of the three NM-IF proteins seen in the parent T-47D5 cell line is no longer observed. The dose-response effects of antiestrogen were examined in T-47D5 cells grown in the presence of estrogen. Similar to what we observed with estrogen dose effects, the abundance of these proteins in the NM-IF fraction was sensitive to alteration by antiestrogen. With as little as 1 nM ICI 164 384 or 0.1 nM OT, significant reductions in the levels of these proteins in the NM-IF were seen (Fig. 6A,B).

Identification of 54-, 45-, and 41-kD Proteins

These proteins were subsequently identified as cytokeratin 8 (54 kD), cytokeratin 18 (45 kD), and cytokeratin 19 (41 kD). Identification was performed by chromatographically enriching for these proteins, isolating spots from 2-D gels, followed by microsequencing of the spots from T-47D human breast cancer cells (unpublished results). These protein bands were confirmed to be the same proteins identified in the NM-IF of T-47D5 human breast cancer cells after column purification of these proteins from T-47D5 cells followed by 2-D gel analysis of fractions (apparent molecular size and pI comparisons) from T-47D, T-47D5, and mixing experiments of the two sets of column fractions from each cell line (Fig. 7). 2-D gel analysis performed on samples with and without estrogen treatment confirmed that the spots identified as CK8, CK18, and CK19 increased in abundance with estrogen treatment as seen in SDS–PAGE (data not shown).

NM-IF Proteins in ER^- Versus ER^+ Cells

To find out if a correlation exists between ER expression and expression of NM-IF proteins, total NM-IF composition was compared between ER^+ and ER^- human breast cell lines. Figure 8 clearly shows a marked difference in total NM-IF composition between ER^+ and ER^- cell lines. In particular, a notable decrease or absence of the cytokeratin 8, 18, and 19 bands was observed in some ER^- breast cell lines.

DISCUSSION

We have identified three NM-IF proteins (CK8, CK18, and CK19) in T-47D5 human breast cancer cells that are regulated by estrogen. The

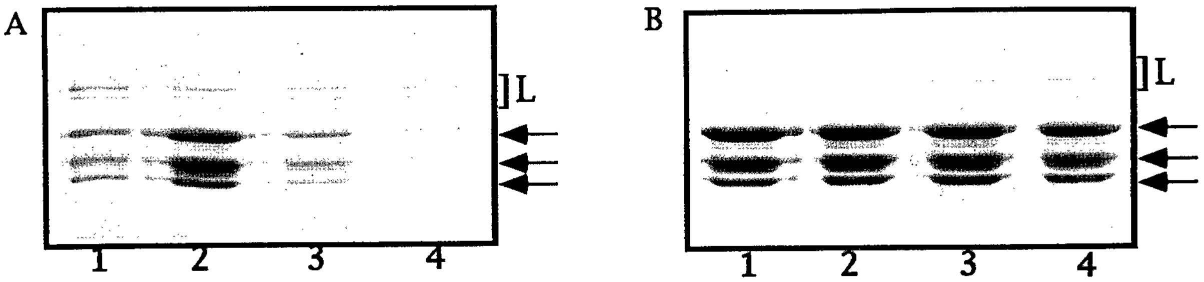
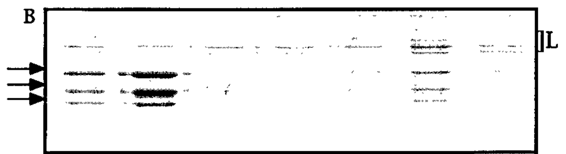


Fig. 5. Effects of antiestrogen on NM-IF proteins in T-47D5 human breast cancer cells. Cells were treated with drug at the appropriate concentration or vehicle control for 72 h before NM-IF isolation. **A:** T-47D5 NM-IF. *Lane 1,* vehicle control; *lane 2,* 10 nM E2; *lane 3,* 100 nM ICI 164 384; *lane 4,* 100 nM 4-monohydroxytamoxifen. **B:** T5-PRF NM-IF. *Lane 1,* vehicle control; *lane 2,* 10 nM E2; *lane 3,* 100 nM ICI 164 384; *lane 4,* 100 nM 4-monohydroxytamoxifen. *Arrows,* 54-, 45-, and 41-kD protein bands; 5 μg protein/lane. L, lamins.

1	2	3	4	5	6	7
100	151+/-37	138+/-30	85+/-18	57+/-16	90+/-11	41+/-10



1	2	3	4	5	6	7
100	166+/-27	62+/-30	49+/-19	70+/-27	67+/-21	64+/-21

Fig. 6. Antiestrogen dose-response effects on NM-IF expression in T-47D5 human breast cancer cells. Cells were treated with antiestrogen at the appropriate concentration or vehicle alone for 72 h before isolation of the NM-IF. **A:** ICI 164 384 dose-response. *Lane 1,* ethanol vehicle; *lane 2,* 10 nM E2; *lane 3,* 0.1 nM ICI; *lane 4,* 1 nM ICI; *lane 5,* 10 nM ICI; *lane 6,* 100 nM ICI; *lane 7,* 1 μM ICI. **B:** 4-Monohydroxytamoxifen dose response. *Lane 1,* ethanol vehicle; *lane 2,* 10 nM E2; *lane 3,* 0.1 nM OT; *lane 4,* 1 nM OT; *lane 5,* 10 nM OT; *lane 6,* 100 nM OT; *lane 7,* 1 μM OT. *Arrows,* 54-, 45-, and 41-kD protein bands; 5 μg protein/lane. L, lamins. Numbers below represent cytokeratin levels as a percentage of control ±SEM. Lamin bands were used as a loading control; n = 3.

abundance of these proteins in the NM-IF fraction is dramatically reduced upon acute withdrawal of estrogen from the cell culture medium, and re-addition of estrogen results in increased levels of these proteins in the NM-IF. The reduced levels of these three NM-IF proteins in the absence of hormone reflect the requirement of estrogen for upregulated expression of these NM-IF proteins (i.e., they are estrogen responsive proteins, or that the association of these proteins with the NM increases upon estrogen treatment). Antiestrogens downregulate the levels of these three NM-IF proteins consistent with an estrogen antagonistic activity of these compounds, suggesting the effect is mediated via the ER.

Chronic estrogen-depleted T-47D5 cells have overcome the requirement of estrogen for growth in vitro, while remaining ER^+. Despite the depletion of estrogen in the cell culture medium the NM-IF fraction from these cells has elevated levels of all three proteins. In the parent T-47D5 cell line addition of estrogen to the cell culture medium results in increased levels of the three NM-IF proteins. In the T5-PRF cells, the addition of estrogen at an equivalent dose does not increase the levels of the three NM-IF proteins. How these cells could have elevated levels of these estrogen regulated proteins in the absence of estrogen remains to be determined, but perhaps the cells are able to activate the ER through pathways other than classical estrogen/ER interactions. Recent research has demonstrated that the ER can be activated in a ligand-independent fashion [Aronica and Katzenellenbogen, 1993; Ignar-Trowbridge et al., 1993] and perhaps the ability to activate the ER in the absence of estrogen would confer a growth advantage to the cells and aid in the development of a hormone-independent phenotype. Our data suggest that as the length of time in estrogen-deplete conditions is increased, there is a concommitant increase in the levels of the 41-, 45-, and 54-kD proteins associated with the NM-IF. Although antiestrogens inhibit the growth of T5-PRF cells, they do not decrease the levels of CK8, CK18, and CK19 in the NM-IF as they do in the parent cell line, suggesting that both estrogen/antiestro-

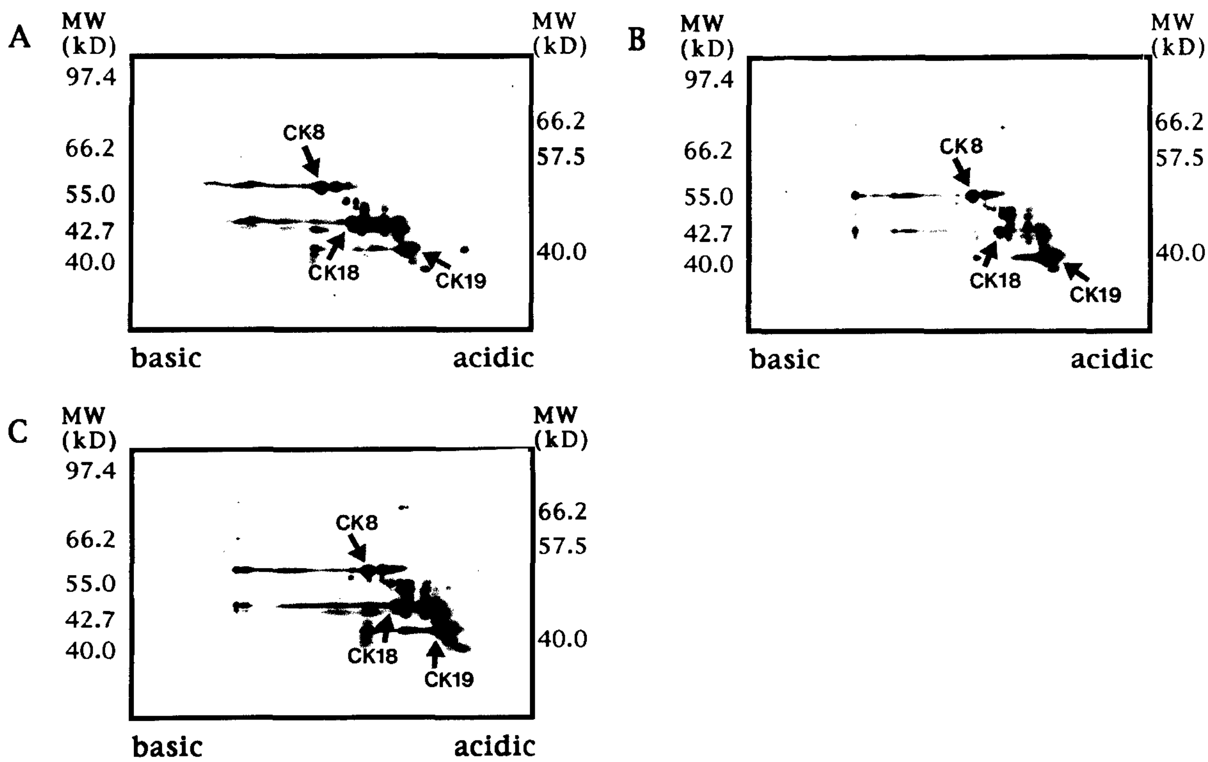


Fig. 7. 2-D gel electrophoresis of T-47D5 and T-47D column fractions. **A:** T-47D5 column fraction. **B:** T-47D column fraction. **C:** T-47D/T-47D5 mix; 15 μg protein/gel.

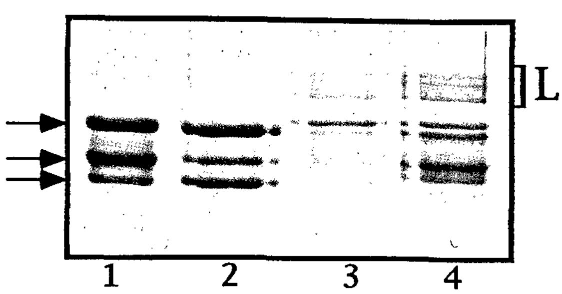


Fig. 8. ER^+ versus ER^- NM-IF protein composition. NM-IF composition was compared between ER^- and ER^+ human breast cancer cell lines. Cells were grown in 5% CM and NM-IF isolated as described in methods. *Lane 1,* T-47D5 (ER^+); *lane 2,* T-47D (ER^+); *lane 3,* MDAMB231 (ER^-); *lane 4,* HBL100 (ER^-). *Arrows,* 54-, 45-, and 41-kD protein bands; 5 μg protein/lane. L, lamins.

gen sensitivity, in terms of regulating the NM-IF levels of these proteins, has been lost in this cell line.

We have purified the p54, p45, and p41 NM-IF proteins and they have been identified by internal microsequencing as cytokeratin 8, 18 and 19, respectively [unpublished results]. That cytokeratins are estrogen regulated in T-47D5 cells is consistent with previous studies. In MCF-7 cells in culture estrogen and antiestrogen treatment both resulted in an apparent increase in cytokeratins [Sapino et al., 1986]. Studies in rat vaginal epithelium have also demonstrated estrogen-induced increases in cytokeratin expression, although antiestrogens did not have a significant effect in these studies [Kronenberg and Clark, 1985]. Androgen, but not estrogen, represses cytokeratin 8 and cytokeratin 18 mRNA expression in the rat prostate, and antiandrogens were shown to reverse this effect [Hsieh et al., 1992].

The ER^- cell lines MDA MB 231 and HBL 100 are estrogen-unresponsive cell lines and have very low levels of cytokeratin 8, 18, and 19 relative to the lamins in their NM-IF. This is consistent with other reports demonstrating in a subset of ER^- cell lines (including MDA-MB-231) an inverse relationship between cytokeratin and vimentin expression [Sommers et al., 1989, 1992]. Significant expression of cytokeratins may therefore be associated with an ER^+ phenotype.

The relationship between tumour growth and cytokeratin expression has been examined, with some studies suggesting a correlation between specific cytokeratin expression and tumour progression [Kannan et al., 1994a,b]. Changes in

the levels and subset of cytokeratin expression have been found with increasing tumour grade [Ferrero et al., 1990; Green et al., 1990]. Several studies have demonstrated enhanced expression of CK19 in tumour tissue, as compared to normal [Cooper et al., 1993; Broers et al., 1988]. Interestingly, in *ras*-transformed prostate cells three NMPs of 41, 46, and 55 kD were found to exhibit significant differences in their expression, as compared with their immortalized controls [Prasad et al., 1993]. These studies suggest that cytokeratins might be a biomarker for tumour stage and perhaps changes in cytokeratin expression are associated with altered tumour phenotype.

Evidence suggests that estrogen-independence in breast cancer cells is associated with changes in expression of a set of estrogen-regulated genes [Brunner et al., 1993; van Agthoven et al., 1993]. van Agthoven and coworkers have shown that in an estrogen-independent ZR-75-1 human breast cancer cell line changes in cell morphology occur along with increases in total cellular levels of cytokeratins 8, 18, and 19 [van Agthoven et al., 1992, 1994]. Although loss of the ER can accompany and/or explain hormone independence, loss of ER does not always occur in the hormone-independent phenotype. Katzenellenbogen and colleagues have demonstrated levels of ER comparable to parent MCF-7 cell lines or greater ER expression in hormone-independent MCF-7 cells obtained through prolonged growth in estrogen-deplete conditions [Katzenellenbogen et al., 1987; Read et al., 1989]. Clarke et al. [1989] isolated a series of hormone-independent MCF-7 sublines that still maintain ER expression at levels comparable to, or greater than, the parent cell line. In our model, continued expression of the ER allows us to examine expression of estrogen responsive genes, under conditions comparable in both the parent and hormone-nonresponsive cell lines.

Our knowledge of the function of the NM-IF proteins is still very limited. Our findings that cytokeratins associated with the nuclear matrix are regulated by estrogen in human breast cancer cells suggests that these structural proteins may be important to estrogen action. Preliminary data suggest that similar patterns of cytokeratin expression can be seen in the NM-IF preparations from some human breast tumour biopsy samples suggesting that these patterns of cytokeratin expression could be relevant to tumour phenotype in vivo.

In conclusion, we have demonstrated that estrogen can regulate the levels of NM-associated intermediate filament proteins. The abundance of these proteins in the NM-IF is increased in T-47D5 human breast cancer cells that are no longer responsive to estrogen in culture. It is hypothesized that such an alteration may result in nuclear architectural changes that could reflect alterations in gene expression associated with a hormone-independent phenotype.

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APPENDIX 11

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IMMUNOHISTOCHEMICAL ASSAY FOR OESTROGEN RECEPTORS IN PARAFFIN WAX SECTIONS OF BREAST CARCINOMA USING A NEW MONOCLONAL ANTIBODY

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SUMMARY

The aim of this study was to evaluate the utility of a new monoclonal antibody (AER311) that targets the oestrogen receptor (ER) in an immunohistochemical assay (IHA) applied to breast cancers. Ninety-seven cases of invasive ductal carcinoma were studied by AER311-IHA using a pressure-cooking antigen retrieval technique applied to formaldehyde-fixed, paraffin-embedded tissue sections; immunostaining was assessed by semi-quantitative scoring (*H* score). There was 80 per cent concordance between the ER status measured by dextran-coated charcoal (DCC) assay and AER311-IHA, with 63/97 (65 per cent) tumours positive and 15/97 (15 per cent) tumours negative by both assays. Of the 12 DCC-positive cases that were negative by AER311-IHA, 11 were borderline positive (3–8 fmol/mg). Similarly, six of seven DCC-negative cases that scored positive by AER311-IHA had only borderline positive *H* scores (<50). When AER311-IHA was compared with 1D5-IHA, there was good concordance in ER status (77 per cent) and a significant correlation (r=0·7, P<0·001) between *H* scores. Nevertheless, the correlation between ER level determined by AER311-IHA and that measured by DCC (r=0·53, P<0·001) was higher than that for 1D5-IHA (r=0·32, P=0·002). AER311-IHA can therefore provide reliable information about the ER status of breast carcinoma on paraffin sections and is an acceptable alternative to other commercially available monoclonal antibodies.

KEY WORDS—oestrogen receptor; breast carcinoma; immunohistochemistry

INTRODUCTION

Determination of oestrogen receptor status is an important parameter in the clinical management of breast cancer.[1–3] The development of new monoclonal antibodies to the oestrogen receptor (ER) and recent advances in immunohistochemical methods have now provided alternatives to the classical biochemical approach to evaluating ER status.[4–6] The immunohistochemical assay (IHA) carries significant advantages with respect to the small amount of tissue required and the potential for assessment of tumour heterogeneity with respect to ER status.[5,6] However, until recently, the IHA was limited to frozen tissue sections by both the characteristics of the available antibodies and the methodologies required. Furthermore, although IHA has also been shown to be capable of predicting response to endocrine therapy,[7] the existence of ER mRNA variants in breast cancer that may be translated into a variety of truncated or abnormal proteins raises the possibility that the target epitope specificity of the specific antibody used may influence the results. Amongst several new ER-specific antibodies, the 1D5 antibody targets the N-terminal AB region of the ER protein, in contrast to the widely used and relatively expensive H222 antibody, which targets a more distal C-terminal region adjacent to the hormone binding domain. It is therefore important to develop and to test antibodies directed against different regions of the ER.

In this paper we describe a sensitive and reliable method to detect ER in routinely processed, formaldehyde-fixed tissue, using a new monoclonal antibody (AER311) for the first time, with a pressure-cooking antigen retrieval technique.

MATERIALS AND METHODS

A cohort of 97 cases of invasive ductal and invasive lobular breast carcinomas was selected for study from the initial 430 cases in the NCIC-Manitoba Breast Tumor Bank. These were chosen primarily on the basis of ER status as determined by the standard dextran-coated charcoal (DCC) assay, to provide a clinically representative range of levels from ER-negative (<3 fmol/mg protein, 22 tumours, 27 per cent), borderline/low ER-positive (3·1–10 fmol/mg, 18 tumours, 15 per cent), moderately ER-positive (10·1–50 fmol/mg protein, 22 tumours, 28 per cent), to high ER-positive (>50 fmol/mg protein, 35 tumours, 30 per cent). Thus the percentage of tumours in each category of ER status within this study cohort approximated to the actual clinical distribution of 5727 cases measured by the Manitoba Provincial Steroid Receptor Laboratory from 1987 to 1995. Additional criteria included good histological tissue quality and the presence of invasive tumour in more than 30 per cent of the surface of the block selected for study.

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All cases had been fixed for 12–18 h in 10 per cent buffered formalin prior to routine embedding in paraffin wax. Immunohistochemistry was performed with AER311, an IgG_{2a} mouse monoclonal antibody raised against SDS-denatured calf uterus ER (Neomarkers, Lab. Vision Corp., U.S.A.), and 1D5, an IgG_1 oestrogen receptor mouse monoclonal antibody (DAKO, Dimension Labs, Canada). At the working dilutions used for AER311, the cost per section was one third of that for 1D5. Sections (5 μm) were cut, mounted on AAS (Sigma)-coated slides, dried overnight at 42°C, and then dewaxed in xylene (5 minutes) and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by immersion in 1 per cent hydrogen peroxide (10 min) followed by washing in running water. The slides were then placed in a metal rack within a domestic pressure cooker, one-third filled with 10 mM citrate buffer solution (pH 6·0). This was brought to the boil on a hot plate (4 min), left to cool (20 min), and then the slides were washed twice in phosphate-buffered saline (PBS).

Normal horse serum (1:20 dilution) was applied (20 min) to block non-specific binding sites, followed by the primary antibody AER311 (1:25 dilution) and incubation overnight in a humid chamber at 4°C. After two washes in PBS, the slides were incubated for 45 min with biotinylated horse anti-mouse IgG (Vector Labs., Burlingame, CA, U.S.A.) at 1:200 dilution. After two additional washes in PBS, the sections were incubated in AB Complex (Elite kit, Vector Labs) at 1:100 dilution (45 min). The label was developed using diaminobenzidine/hydrogen peroxide (4 min). After another wash in running tap water, the colour was enhanced by incubation in 1 per cent copper sulphate (10 min). After a final wash, sections were counterstained lightly with 2 per cent methyl green, washed, dehydrated, and coverslipped. The protocol for the IHA with the 1D5 antibody differed only in the primary antibody. Appropriate negative controls were included with all experiments and positive standards included four tumours that were selected to provide a range of ER levels from high positive (>50 fmol/mg) to negative (<3 fmol/mg).

The cases were assessed without knowledge of the clinical and biochemical data or of the primary antibody, using a semi-quantitative H-score system applied to coded slides.[5] Slides were scored by two observers (NMP and PHW) and differences were resolved by agreement. In each case, five representative fields (×40 objective on a Leitz DMRBE microscope) within each section were selected for assessment. Brown immunoreactivity of tumour cell nuclei was taken as positive and the proportion of negative tumour cells (P_0), and of those staining at low (P_1), moderate (P_2) or high (P_3) levels of intensity, was scored. The H score for each region was then calculated as $H=[P_1+(2\times P_2)+(3\times P_3)]\times 100$ and the mean overall H score was calculated for the tumour section. A tumour was considered 'ER-positive' by DCC assay if the ER level was greater than 3 fmol/mg protein. Meanwhile, a tumour was designated oestrogen receptor-positive by IHA if the H score was higher than 35 (AER-311) or higher than 50 (1D5).

RESULTS

Positive immunoreactivity of nuclei was seen in both malignant and occasional normal epithelial cells in sections stained by the two ER-IHA methods (Fig. 1), with lymphoid and inflammatory components, when present, serving as an internal negative control. A small number of cases showed prominent cytoplasmic staining with both antibodies, but this was not scored when assessing the ER status. Similar results were also obtained with a microwave antigen retrieval method, but the pressure cooker method was found to be more efficient for multiple sections.

Of the 97 cases examined, 63 were ER-positive and 15 were ER-negative by both DCC and ER-IHA-311; results were thus similar in 80 per cent of cases. Chi-squared analysis showed a value of 21·81, $P<0·001$ (Table I). Of the remaining 19 cases, 12 (12 per cent) were positive by DCC but negative with AER-IHA and seven (7 per cent) were negative by DCC and positive with AER311-IHA. However, the DCC levels of 11 cases of the former were between 3 and 8 fmol/mg protein, which may be considered borderline positive and six of the seven cases that were scored as false positive by IHA had an H score of less than 50. The AER311-IHA result was discordant in 7/22 (32 per cent) ER-DCC-negative tumours (<3 fmol/mg) and 12/75 (16 per cent) ER-positive tumours. The latter group included 11/18 low positive (3–10 fmol/mg), 1/22 moderately positive (10–50 fmol/mg) and 0/35 high positive (>50 fmol/mg) by ER-DCC. The use of alternative cut-off points above or below 35 (20 or 50) had relatively little influence on the concordances between the ER-IHA-311 and DCC assays (79 per cent and 74 per cent).

To compare the AER311 antibody with another established antibody, 1D5, adjacent serial sections were stained and similar areas were selected for H-score assessment. As with the AER311-IHA, there was significant agreement between ER status determined by 1D5-IHA and DCC (83 pere cent). The ER H-score level of individual tumours ranged from 0 to 200 with AER311 and up to 237 with the 1D5 antibody, in these parallel immunohistochemistry assays. Amongst the subgroup of ER-DCC-positive tumours, the mean H scores for AER311-IHA and 1D5-IHA were 82 and 114 respectively. There was also a strong correlation ($r=0·7$, $P<0·001$) between the ER level derived from ER-IHA with the two antibodies (Fig. 2). However, 26 tumours were significantly discordant (H-score difference >50) and in 25/26 of these IHA discordant tumours the 1D5 H score was higher than the AER-311 H score. Further statistical analysis showed a better correlation between DCC and AER311-IHA ($r=0·53$, $P<0·001$) than between DCC and 1D5-IHA ($r=0·32$, $P=0·002$) (Fig. 3A and 3B). Using the empirical H score values of 20, 35, and 50 as cut-off points, the sensitivity and specificity of the 1D5 antibody ranged from 92, 91, 87 and

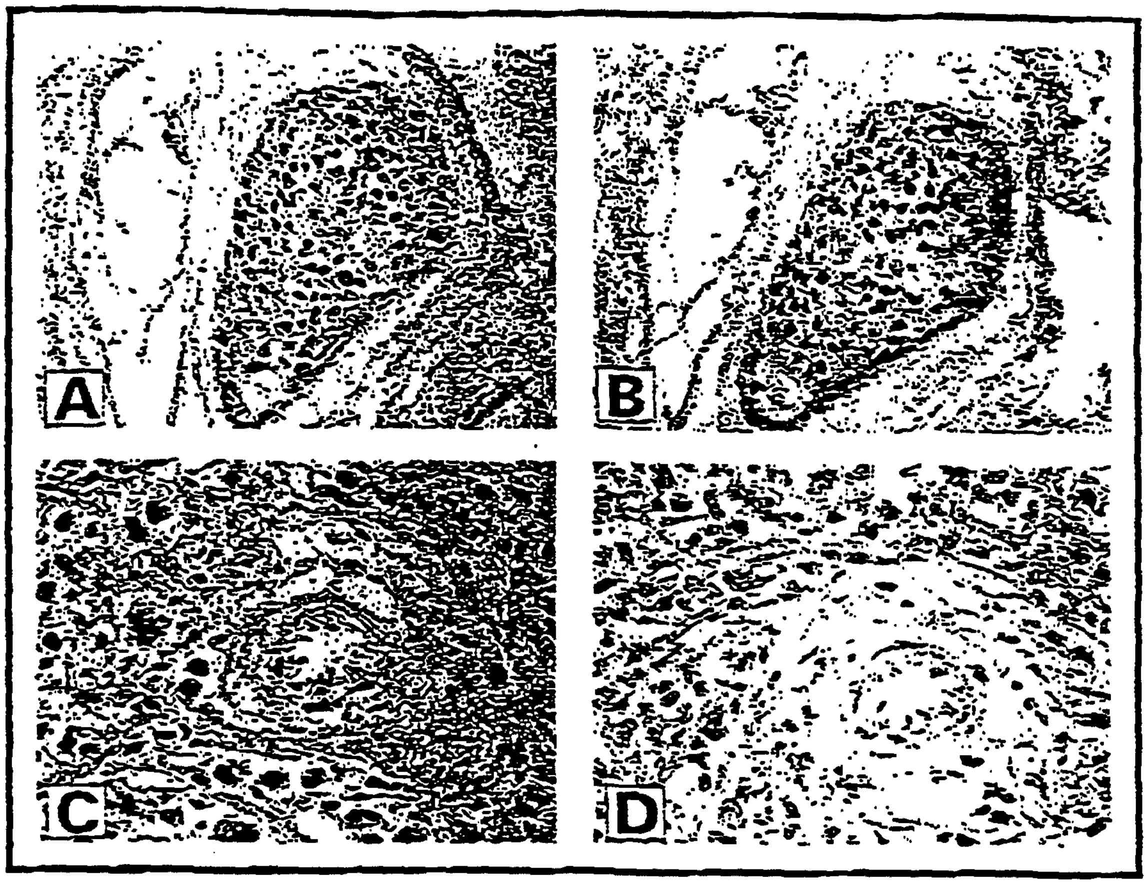

Fig. 1 Examples of immunohistochemical detection of oestrogen receptor by 1D5 (left panels, A and C) and AER-311 (right panels, B and D) antibodies. The upper panels illustrate the detection of similar levels of oestrogen receptor in the intraductal component of the same ER+/PR+ tumour stained with each antibody (case 93. ER 94. PR 98 by DCC), while the lower panels illustrate a significant difference in the IHA signal with each antibody within the invasive component of another ER−/PR+ tumour (case 61. ER 0. PR 11·8 by DCC)

Table 1 --Concordance between AER311 immunohistochemical analysis and biochemical DCC on paraffin wax sections

	DCC+	DCC−	Totals
IGA+	63 (65%)	7 (7%)	70
IHA −	12 (12%)	15 (15%)	27
Totals	75	22	97

χ^2=21·81; degree of freedom=1; P<0·001.

55. 68. 86 respectively, while the sensitivity of AER311 was 85. 84. 68 and the specificity was 59. 68. 95 at each cut-off.

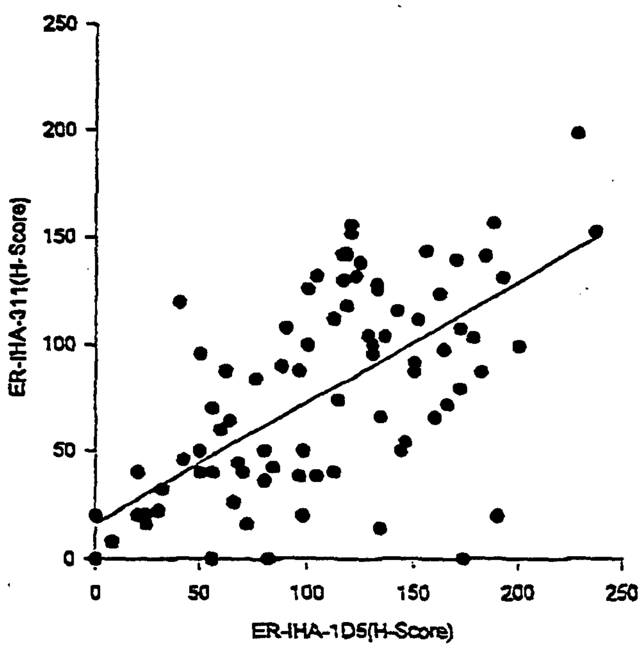

Fig. 2 Correlation between ER-IHA-311 and ER-IHA-1D5 in 97 breast cancers (r=0·7), P<0·001)

DISCUSSION

We have shown that immunohistochemical assay with a new monoclonal antibody (AER311) allows the accurate assessment of ER in archival paraffin sections from human breast cancer. In terms of determination of ER status, the concordance between the AER311-IHA and the classical ER-DCC assay is similar to that achieved in other studies that have compared IHA in paraffin sections with the DCC assay.[9,10] However, in terms of ER level, the AER311-IHA correlates better with the ER-DCC level than IHA performed with the 1D5 antibody in this series of selected tumours.

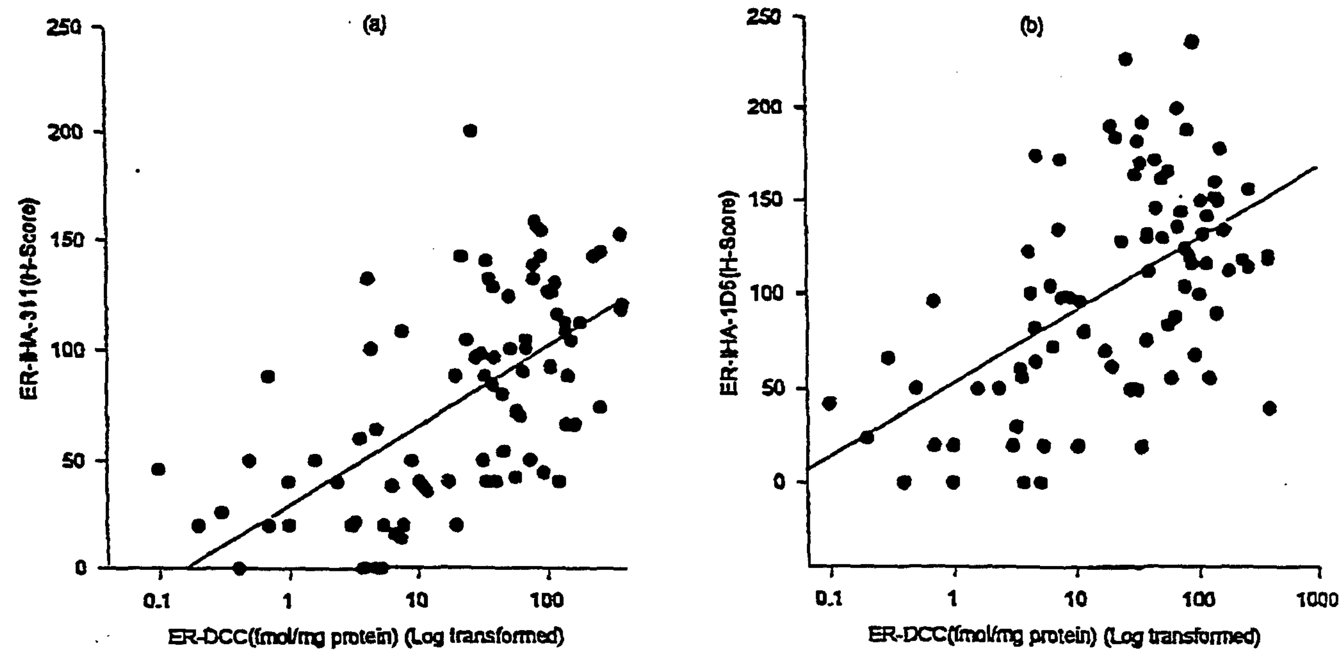

Fig. 3—Correlation between the ER-DCC assay and ER-IHA-311 (A) and ER-IHA-1D5 (B) in 97 breast cancers ($r=0.53$, $P<0.001$ and $r=0.32$, $P=0.002$, respectively)

The cut-off point to resolve ER status was determined retrospectively for each antibody and this will need to be confirmed in a prospective study and on the basis of clinical response to endocrine therapy. However, a comparison with the DCC ER status in these tumours and previous experience with other antibodies suggests that the appropriate cut-off for *H*-score assessment may lie in the range between 20 and 50. Comparing assays with a positive cut-off point of 35 for the AER311-IHA and the established cut-off of 3 fmol/mg protein for the ER-DCC assay, there was agreement in 80 per cent of cases, which is similar to but lower than that in other recent studies comparing ER-DCC status with ER-IHA in paraffin wax sections.[9,10] In ten comparable studies reviewed by Andersen, the median agreement was higher overall (88 per cent), but the concordance was similar (82 per cent) if only the larger studies with more than 70 cases were considered.[11] However, any comparison between studies should consider the composition of the tumour series in terms of ER status and level, and this is not always clear. For example, as in other studies,[12] in almost all of our cases where the IHA yielded a false-positive result, the tumours possessed borderline ER-DCC levels; this ER-DCC group represented 20 per cent of this selected study cohort.

We chose to compare AER311 with the 1D5 antibody because the latter is well characterized, produces results in IHA that correlate well with the widely used H222 antibody, and is distinct from AER311 in that it targets the N-terminal region of the ER protein. Direct comparison of the two antibodies showed a highly significant correlation in overall determination of ER status. Nevertheless, AER311 showed a better correlation than 1D5 with the ER level determined by DCC assay ($r=0.53$ vs. $r=0.32$).

Similar discrepancies between ER-DCC and ER-IHA have been noted in previous studies in which other antibodies were used.[10,13,14] A degree of variation between the ER assessment provided by the functional DCC assay and the essentially structural immunohistochemical approach is not unexpected. Variability in tissue composition between samples may also contribute to IHA versus DCC assay differences, but not to discrepancies between two parallel immunohistochemistry assays. Despite the strong overall association between the results with AER311 and 1D5, there were cases which demonstrated substantially different *H*-score values. Although this could be attributable to different inherent affinities between the two antibodies, another possible explanation may lie in the fact that these monoclonal antibodies target different parts of the ER. AER311 recognizes an epitope that maps between amino acids 495 and 595 within the E/F domain at the C-terminal of the ER protein.[15] Thus, the AER311-IHA will only detect full length ER proteins with an intact hormone binding domain region. In contrast, the 1D5 antibody recognizes an epitope at the other end of the receptor protein, in the N-terminal A/B region.

This difference in target epitope specificity may be important in view of the many different ER mRNA variants that have been described in breast cancers.[16,17] Amongst the several different 'truncated' ER variant mRNAs, some, such as the ER clone 4 variant, appear to be highly prevalent in ER-positive tumours.[18] Several 'deleted' variants also exist and in some cases they are thought to encode proteins with either constitutively active (exon d5 variant) or dominant negative (exon d7 variant) activities.[19,20] If these ER mRNA variants are translated *in vivo*, then many of the predicted proteins would be recognized by the 1D5 antibody (in addition to normal wild-type ER), but the alteration in the reading frames, resulting from the truncation or deletion, would yield proteins that would not be recognized by the AER311 antibody. It is therefore interesting to note that

of the 25 per cent of tumours that were significantly discordant in our series, almost all demonstrate high *H* scores by ER-IHA-1D5 relative to ER-IHA-311. Further investigations of the patterns of ER mRNA expression in those tumours which show discrepancies between ER-specific antibodies directed against different parts of the receptor may be of value in assessing the influence of ER variants on the determination of ER status.

In conclusion, we believe that AER311 antibody is a good, less costly alternative to other commercially available antibodies, H222 or 1D5, for assessment of ER status by IHA on paraffin-embedded sections of routinely processed, formaldehyde-fixed breast tumour tissue.

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APPENDIX 12

EXPRESSION OF ESTROGEN RECEPTOR VARIANT mRNAs AND DETERMINATION OF ESTROGEN RECEPTOR STATUS IN HUMAN BREAST CANCER

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ABSTRACT

Estrogen receptor (ER) status of breast cancer can be assessed by immunohistochemical assay (IHA), however we have previously observed that ER-IHA levels can be inconsistent between N-terminal and C-terminal targeted antibodies. To address the hypothesis that this discrepancy is attributable to expression of ER variant mRNAs encoding truncated ER-like proteins we have studied 39 'IHA-consistent' and 24 'IHA-inconsistent' breast tumors by reverse-transcription-polymerase-chain-reaction (RT-PCR) to examine the expression of multiple exon-deleted (D-ER) variant mRNAs and the truncated ER clone 4 variant mRNA. ER variants D7-ER, D4-ER, D3-4-ER, and D4-7-ER were detected at similar frequencies in both groups. However, ER variants D2-3/7-ER, D2-3-4-ER ($P<0.05$) and D-3-7-ER ($P<0.01$) which encode putative short ER-like proteins that might be recognized only by an N-terminal targeted antibody, were preferentially detected in 'inconsistent' cases. ER clone 4 mRNA expression was also higher in inconsistent tumors ($P<0.001$). Further analysis showed that whereas overall prevalence of ER variant mRNA's was similar in both tumor groups, occurrence of the subset of variant mRNAs encoding putative truncated proteins was also higher in 'IHA-inconsistent' tumors ($P<0.05$). These data suggest that ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER-IHA levels determined using N- or C-terminal targeted antibodies.

INTRODUCTION

Estrogen receptor (ER) determination is an important parameter in the clinical management of breast cancer.[1,2] Until recently ER content was assessed principally by ligand-binding techniques such as dextran-coated charcoal (DCC) or sucrose gradient assays. Now, with the development of several antibodies able to recognize ER protein, immunohistochemical assay (IHA) has become an alternative approach to determine ER status of breast tumors and to predict endocrine response in breast cancer.[3,4] The ER-IHA approach has significant advantages including the potential for parallel assessment of tumor cell content and heterogeneity of ER expression. However it differs from 'traditional' methods in that ER activity is defined by structural rather than functional criteria.

ER-IHA in tissue sections has been successfully achieved by several different antibodies, including 1D5, H222, and AER311 which are able to recognize different epitopes within particular domains of the ER protein (Figure 1).[3-5] However, we and others have previously observed that the ER-IHA results from some tumors are discordant between different antibodies that are able to recognize either the NH_2 or the COOH terminals, with a tendency to higher signals with NH_2 terminal targeting antibodies.[4,5] While these differences might relate to different antibody affinities, another explanation lies in the existence of ER variants. Beside the wild-type ER mRNA transcript, several ER variant mRNAs have been described in both normal and cancer tissues.[6-14] Most of these variants are suspected to result from alternative splicing of WT-ER mRNA, and consist of exon-deleted and truncated variants.[6,8] Figure 1 shows some of the putative proteins encoded by these variants and illustrates that whereas some of these altered proteins may still possess both NH_2 and COOH terminal epitopes of the wild type (WT) protein, others will be truncated and lack the COOH terminal as a result of an exon deletion that introduces a shift in the reading frame. In addition to exon-deleted ER mRNA variants, several truncated variants have been described, amongst which the ER clone 4 variant

is highly prevalent in breast tumors.[8] The sequence of this variant mRNA corresponds to WT-ER exon 1 and 2 juxtaposed to Line 1 related sequences, and in-vitro analysis shows that it encodes a putative ER-like protein missing the C-terminal extremity.

To address the hypothesis that discrepancies observed by IHA using 1D5 and AER311 antibodies in breast tumors could result from particular ER variant expression, we investigated 39 'IHA-consistent' and 24 'IHA-inconsistent' breast tumors for the most prevalent exon-deleted ER variant mRNAs and in parallel for the level of ER clone 4 truncated variant mRNA expression by two RT-PCR assays that we have recently developed to assess multiple ER variants in breast cancer tissues.[15, 16]

MATERIALS AND METHODS

Human Breast Tissues and ER status determination

The study was carried out on 63 cases of invasive ductal and invasive lobular breast carcinomas obtained from the NCIC-Manitoba Breast Tumor Bank.[17] These cases correspond to the ER positive subset of a series of 97 tumors previously studied by IHA.[5] In all cases, the specimens had been rapidly frozen at -70 °C as soon as possible after surgical removal. Subsequently, a portion of the frozen tissue from each case was processed routinely to create formalin-fixed- paraffin-embedded tissue blocks that were matched and orientated relative to a corresponding frozen tissue block. Paraffin sections were previously analyzed by IHA using 1D5 (Dako, Dimension Labs, Canada) and AER311 (Neomarkers, Lab. Vision Corp., USA) ER-monoclonal antibodies.[5] In each case, immunohistochemical staining was assessed, without knowledge of the ER-DCC status or antibody used, by a semi-quantitative H-Score system (range 0 to 300) for both antibodies and in the same regions on adjacent serial sections. When a difference of H-Score values between the two antibodies

was >50, tumors were classified as "inconsistent". When the difference of H Score values was lower than 50, the tumors were considered as "consistent". Within the inconsistent tumor group (24 cases), 8 tumors were low ER-positive (3-10 fmol/mg protein, 33 per cent), 6 tumors were middle ER-positive (11-50 fmol/mg protein, 25 per cent) and 10 were high ER-positive (>50 fmol/mg protein, 42 per cent), as determined by ligand-binding assay. Within the consistent tumor group (39 cases), 6 cases were low ER-positive (15 per cent), 12 cases were middle ER-positive (31 per cent) and 21 were high ER-positive (54 per cent). Overall, in the 'inconsistent' and 'consistent' groups the mean$^{(SD)}$ ER was $42.5^{(86.2)}$ versus $57.0^{(89.2)}$ while the mean$^{(SD)}$ PR was $23.5^{(68.7)}$ versus $29.1^{(75.4)}$ respectively.

Extraction of mRNA and Reverse Transcription

For each case, a specific face of a frozen tissue block that matched the corresponding face of the paraffin block previously studied by IHA was selected.[17] Total RNA was extracted from histologically defined regions within 20 µm cryostat sections of frozen tissue using a small scale RNA extraction protocol (Trireagent, MRCI, Cincinnati, OH, USA) as previously described.[18] Reverse transcription reactions were performed in triplicate in a final volume of 15 µl[13, 15] and 1 µl of the reaction mixture was taken for subsequent PCR amplification in either 'long-range-PCR' or 'triple-primer PCR' assays described below.

Analysis of prevalence of ER variant mRNAs

Prevalence of ER variant mRNAs within breast tumor samples was assessed by PCR analysis performed by a 'long-range-PCR' assay as previously described.[15] The primers used consisted of 1/8U primer (5'-TGCCCTACTACCTGGAGAACG-3'; sense; located in WT-ER exon 1) and 1/8L primer (5'-GCCTCCCCCGTGATGTAA-3'; antisense; located in WT-ER exon 8). This primer set allowed

amplification of 1381 bp fragment corresponding to WT-ER mRNA and all deleted or inserted ER variant mRNAs containing exon 1 and exon 8 sequences. PCR amplifications were performed in a final volume of 10 μl, in the presence of 10 nM of dCTP [α-^{32}P] (ICN Pharmaceuticals, InC., Irvine, CA), 4 ng/μl of each primer and 1 unit of Taq DNA polymerase (Promega, Madison, WI). Each PCR consisted of 40 cycles (1 minute at 60°C, 2 minutes at 72°C, 1 minute at 94°C) using a thermalcycler (MJ Research PT100, Fisher Scientific, Ottawa, ON). After PCR, 2 μl of the reaction was denaturated in 80% formamide buffer and the PCR products were separated on 3.5% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and autoradiographed for 18 hours. Identities of specific bands were then confirmed by reference to size markers, subcloning and sequencing.[13]

Quantification of ER clone 4 mRNA expression

Quantification of clone 4 mRNA expression was performed using a 'triple-primer PCR' assay as previously reported.[16] Briefly, three primers, that consisted of E2U (5'-AGGGTGGCAGAGAAAGAT-3', sense, located in WT-ER exon 2), E3L (5'-TCATCATTCCCACTTCGT-3', antisense, located in WT-ER exon 3) and C4L (5'-GGCTCTGTTCTGTTCCATT-3', antisense), were used during PCR, performed in the presence of dCTP [α-^{32}P]. These primers allowed the co-amplification of a 281 bp and a 249 bp fragment corresponding to WT-ER and clone 4 truncated ER variant mRNAs, respectively. PCR products were separated by PAGE. Following electrophoresis, gels were dried and autoradiographed. Autoradiographs were analyzed with a video-densitometry system and quantitated using MCID M4 software (Imaging Research Inc., St. Catherines, ON). The signal corresponding to ER clone 4 was measured relative to expression of the corresponding WT-ER and expressed as a percentage relative to

a reference standard (an ER positive tumor sample) in order to reduce any variation due to signal intensity in different gels. ER clone 4 expression was determined from the mean of three independent RT-PCR assays performed without knowledge of the IHA status. Means obtained from the 24 'IHA-inconsistent' tumor samples were then compared with those found in the 39 'IHA-consistent' tumor samples using the Mann-Whitney rank sum test (two-sided).

RESULTS

Detection of exon-deleted ER variant mRNAs within consistent and inconsistent tumors

Prevalence of exon-deleted ER variant mRNAs was investigated within 63 breast tumors, previously studied by IHA using 1D5 and AER311 antibodies[5] and subsequently classified as 'IHA-consistent' (39 cases) or 'IHA-inconsistent' (24 cases) as illustrated in Figure 2. 'Long-range' RT-PCR assay using primers annealing with exon 1 (1/8U) and exon 8 (1/8L) sequences first allowed assessment of the most prevalent exon-deleted variant mRNAs in comparison with the co-amplified WT-ER mRNA, as described previously.[15] Several different PCR products were observed within the set of tumors studied (Figure 3) that have previously been shown to correspond to the WT-ER (1381 bp) and ER variant mRNAs deleted in exon 7 (D7-ER, 1197 bp), exon 4 (D4-ER, 1045 bp), both exon 3 and 4 (D3-4-ER, 928 bp), exons 2,3 and 7 (D2-3/7-ER, 889 bp), both exons 4 and 7 (D4/7-ER, 861 bp), exons 2,3 and 4 (D2-3-4-ER, 737 bp), and within exon 3 to within exon 7 (D-3-7-ER, 580 bp), respectively.[15] Results obtained for 'IHA-consistent' and 'IHA-inconsistent' tumor subgroups are summarized in Table 1. D7-ER, D4-ER, D3-4-ER and D4/7-ER variant mRNAs were detected at the same frequency in both subgroups. However, D2-3/7-ER, D2-3-4-ER and D-3-7-ER mRNAs were preferentially detected in 'IHA-inconsistent' tumors. This increased prevalence reached statistical significance for both D2-3-4-ER and D-3-7-ER mRNAs ($p<0.05$ and $p<0.01$). Given that the D7-ER

variant was detected uniformly (>90%) in both subgroups we chose to assess the remainder of the variant mRNAs that were not uniformly detected (i.e all variants excepted D7-ER). These were then considered with respect to the putative ER-like protein they should encode and classified further into two subgroups. In frame variants (ER $V.^{IF}$) comprised those whose sequence modification did not introduce a shift in the reading frame and that could encode proteins theoretically recognized by both 1D5 and AER-311 antibodies (D4-ER and D3-4-ER variant mRNAs). Out of frame variants (ER $V.^{OF}$) comprised variants encoding proteins theoretically only recognized by 1D5 antibody (D4/7-ER, D2-3/7-ER, D2-3-4-ER and D-3-7-ER). ER $V.^{IF}$ were detected in 12 (31%) and 8 (33%) 'IHA-consistent' and 'IHA-inconsistent' tumors, respectively. At the same time, ER $V.^{OF}$ were detected in only 6 (15%) 'IHA-consistent' compared to 10 (42%) 'IHA-inconsistent' tumors, respectively ($P<0.05$, Chi squared analysis).

Quantification of Clone 4 mRNA expression

Expression of a prevalent truncated ER mRNA variant, the ER-clone 4 variant, which is also suspected to encode a truncated ER-like protein, was then analysed by 'triple-primer RT-PCR' using 3 primers to allow the co-amplification of WT-ER mRNA together with clone 4 variant mRNA, as described previously.[16] Typical results from 'IHA-consistent' and 'IHA-inconsistent' tumors are shown figure 4. PCR products (bands of 281 bp and 249 bp) corresponding to WT-ER and ER clone 4 mRNAs were observed in all tumors. Using the Mann-Whitney rank sum test (two-sided), the relative expression of clone 4 truncated variant ER mRNA to WT-ER mRNA was also found to be significantly ($P<0.01$) higher in 'IHA-inconsistent' tumors (median =80.4%, SD =18.7%) versus 'IHA-consistent' tumors (median = 62.4%, SD = 14.4%). (Figure 5)

DISCUSSION

Using PCR-based approaches that allow the investigation of the prevalence of different exon-deleted and truncated ER variant mRNAs within breast tumor samples, we have investigated ER variant mRNA expression within 63 breast tumors which presented similar ('IHA-consistent') or different ('IHA-inconsistent') results when assessed for ER expression by IHA performed with an antibody (1D5) recognizing the N-terminal as compared to an antibody (AER311) targeting the C-terminal of the ER protein. We have found that while variants such as D7-ER, D4-ER, D3-4/ER and D4/7-ER are detected at the same frequency in 'IHA-inconsistent' and 'IHA-consistent' breast tumors, other variants including D2-3/7-deleted, D2-3-4-ER and D-3-7-ER are preferentially detected in 'IHA-inconsistent' cases. This difference between subgroups was statistically significant for two of these variants, exon-2-3-4-deleted ER and exon-3-7-ER. Both of these two variant mRNAs, possess sequence modifications that introduce a shift in the WT-ER coding sequence, that would encode ER-like proteins containing the N-terminal TAF-1 transactivation domain but missing all the C-terminal extremity of WT-ER protein (figure 1). These putative variant ER proteins would therefore theoretically be recognized by 1D5 antibody but not AER311 antibody. Furthermore, detectable expression of the subset of variant mRNAs able to encode truncated ER-like proteins (except the uniformly prevalent D7-ER variant that was detected in all but 6 tumors out of the 63 studied) was significantly higher in the 'IHA-inconsistent' tumor group. In contrast, detectable expression of variants encoding in-frame proteins that should be recognized by both antibodies was no different between tumor subgroups. Taken together, these results are in keeping with the hypothesis that ER variant mRNAs encoding truncated ER proteins may participate in the synthesis of ER-like proteins differentially recognized by 1D5 and AER311 antibodies. This assumption is also further supported by the results obtained using a quantitative PCR-based approach applied to the same tumors, which

indicate that 'IHA-inconsistent' tumors also possess significantly higher levels of ER clone 4 truncated variant relative to WT-ER compared to 'IHA-consistent' tumors.

Until the development of antibodies that are specific for individual ER variant proteins, the premise that proteins encoded by ER variant mRNAs may directly interfere with ER immunodetection and determination of ER status by IHA remains to be proven. It is clear from in-vitro laboratory studies that ER variants can encode proteins which possess a variety of dominant negative, positive or undetectable activities when tested for their ability to interfere with transactivation of classical ER enhancer sequences/elements.[6-9,19] Thus although we and others [4,5] have observed a relative increase in N terminal signal that may correspond to increased truncated ER proteins, the functional implications in terms of response to endocrine therapy will depend on the nature of the specific ER variant activities in a given tumor.

While a good correlation between ER-DCC and ER-IHA is often found, approximately 20% of cases are discordant.[5] It is believed that the cause of this discordance is multifactorial and both ER-DCC positive/IHA negative and ER-DCC negative/IHA positive cases have been attributed overall to tumor heterogeneity, sampling, variable frozen tissue handling and formalin fixation.[20] However an explanation for discordant results is not always apparent in specific cases.[20] Thus although recent studies have shown that immunodetection using 1D5-IHA can accurately predict endocrine response of breast cancer,[21] the relative predictive value of ER-DCC versus ER-IHA is still under debate.[20-22] In the light of our results, and laboratory evidence to suggest that ER variant proteins encoded by ER variant mRNAs may participate in endocrine response,[6-14] it may be important to assess ER variant expression in future studies concerning ER-IHA status and response to endocrine therapy.

Interestingly, the two exon-deleted ER variant mRNAs whose expression was shown here to be correlated to inconsistent results by IHA (i.e D2-3-4-ER and D-3-7-ER), have not been detected

until recently.[15] However our previous studies[5] indicate that expression of these variants may be associated with high grade tumors and high ER level respectively.[15] Similarly, we have shown that a higher level of ER clone 4 mRNA expression correlates with tumor progression and poor prognosis.[16,24] This suggests that these ER variant mRNAs may not only contribute to discrepant IHA results but alteration of their expression is associated with tumor progression.

In conclusion, we have found a significant correlation between expression of certain ER variant mRNAs and inconsistent IHA results following assessment and comparison of ER expression with antibodies directed to either N or C terminal epitopes in human breast cancer. This data adds to the growing body of evidence that suggests that ER variants may be translated in-vivo into ER-like proteins. [5,25,26] Finally, these results suggest that ER variant expression may be an important parameter to consider in the determination of ER status in human breast cancer.

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FIGURE LEGENDS

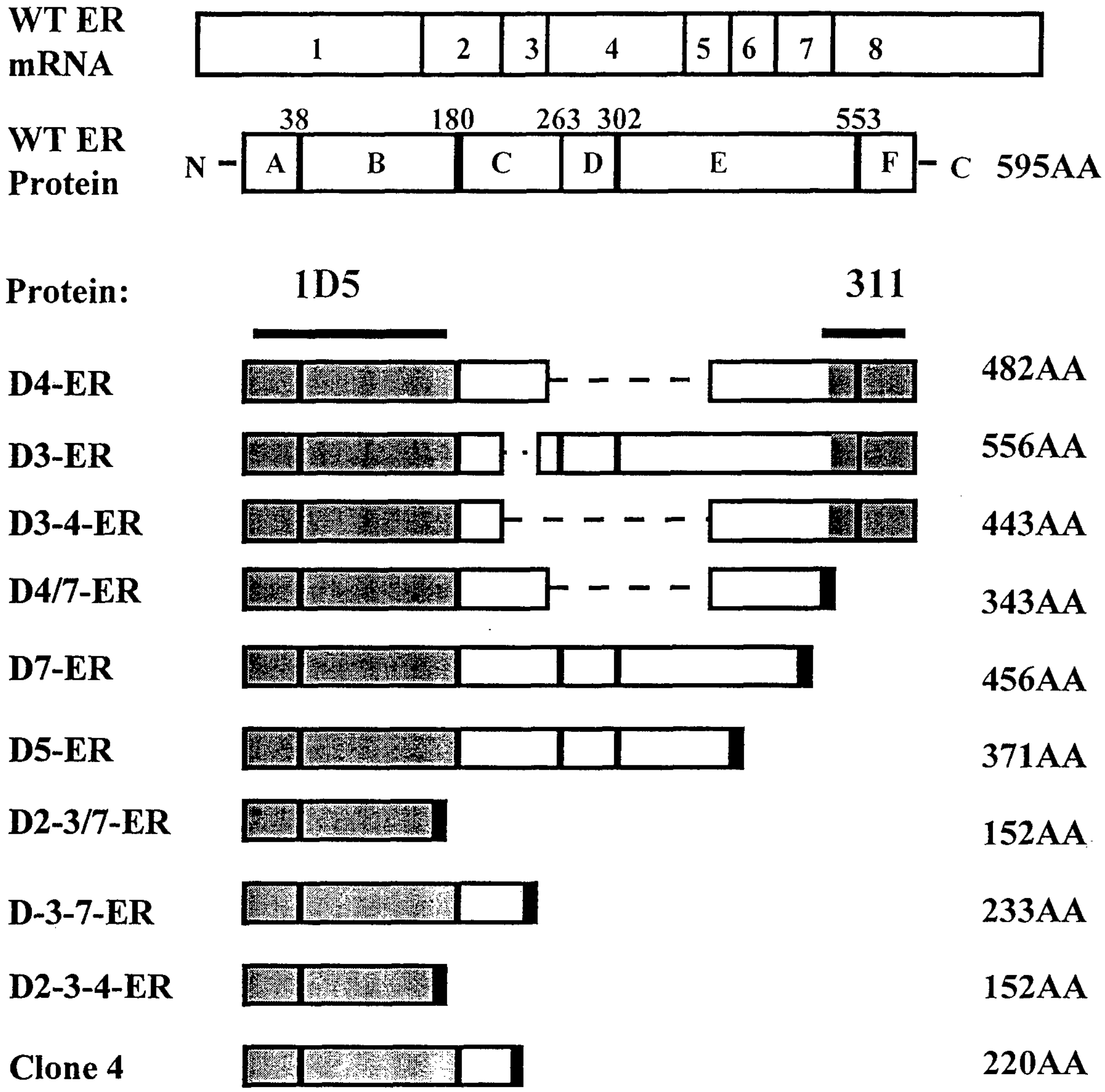
Figure 1. Schematic presentation of WT-ER protein and the predicted proteins encoded by ER variant mRNAs. ER protein contains A-F functional domains. Region A/B of the receptor is implicated in trans-activating function (TAF1). The DNA binding domain is located in the C region. Region E is implicated in hormone binding and another transactivating function (TAF2). WT-ER reading frame is conserved in ER variant mRNAs deleted in exon 4, exon 3 and in both exon 3 and 4. Encoded proteins from D4-ER, D3-ER and D3-4-ER respectively, are similar to WT-ER (open box) but miss some internal amino acids (hatched line). Simple deletion of exon 7 or exon 5, multiple deletion of exon 4 and exon 7, exons 2, 3 and 7, exons 3 and 7, and exons 2,3 and 4 introduce a shift in the ER-WT reading frame. The resulting proteins, D7-ER, D5-ER, D4/7-ER, D2-3/7-ER, D-3-7-ER and D2-3-4-ER, respectively, are therefore similar to WT-ER (open box) but are truncated of the C terminal WT region (black box, indicating amino acids different from WT-ER). Clone 4 protein is encoded by an ER variant mRNA containing WT-ER exon 1 and exon 2 juxtaposed with line 1 related sequences. Clone 4 protein is similar to WT-ER (open box) but miss the C terminal. Grey area represent regions of the protein that are theoretically recognized by 1D5 or AER311 antibodies.

Figure 2. Graph to show the difference in H-score (IHA-1D5 - IHA-311) for each of 63 tumors showing the basis for classification into 'IHA-consistent' (<50 H-score difference, white bars) and 'IHA-inconsistent' tumors (>50 H-score difference, black bars).

Figure 3. Comparison of exon-deleted ER variants expression between 'IHA-consistent' (1-4) and IHA-inconsistent' (5-9) breast tumors. Total RNA was extracted from inconsistent and consistent tumors, reverse transcribed and subsequently amplified by PCR as described in the "Materials and Methods" section. PCR products were separated on PAGE and visualized by autoradiography. Bands migrating at 1381 bp, 1197 bp, 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp and 580 bp were identified by isolation and sequencing as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3 and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2, 3 and 4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. M: molecular weight marker (ϕ X174, Gibco BRL, Grand Island, NY).

Figure 4 Expression of clone 4 variant ER mRNA in tumors representative of 'IHA-inconsistent' (lanes 1-5) and 'IHA-consistent' (lanes 6-10) tumor subgroups. RNA extracted from tumors was analyzed by triple-primer PCR as described above. Upper and lower arrows show wild-type and clone 4 corresponding signals, respectively.

Figure 5 Comparison of the relative expression of ER clone 4 variant mRNA in 'IHA-inconsistent' breast tumors and in 'IHA-consistent' breast tumors. For each sample, the mean of three independent measures of clone 4 expression was expressed as a percentage of the corresponding wild-type ER signal. The difference between two groups is statistically significant ($P<0.01$, Mann-Whitney Rank Sum Test, two sided).


WT ER
mRNA
1
2
3
4
5
6
7
8
WT ER
Protein
N
38
180
263
302
553
A
B
C
D
E
F
C
595AA
Protein:
1D5
311
D4-ER
482AA
D3-ER
556AA
D3-4-ER
443AA
D4/7-ER
343AA
D7-ER
456AA
D5-ER
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152AA
Clone 4
220AA

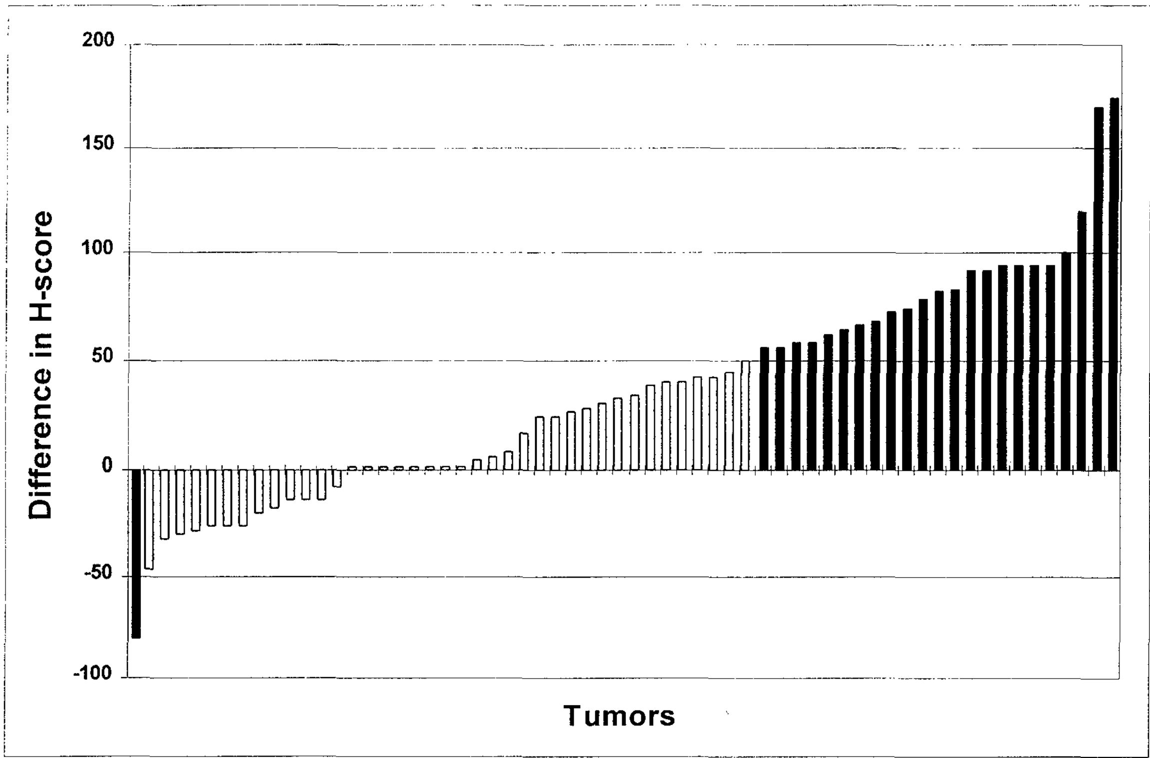
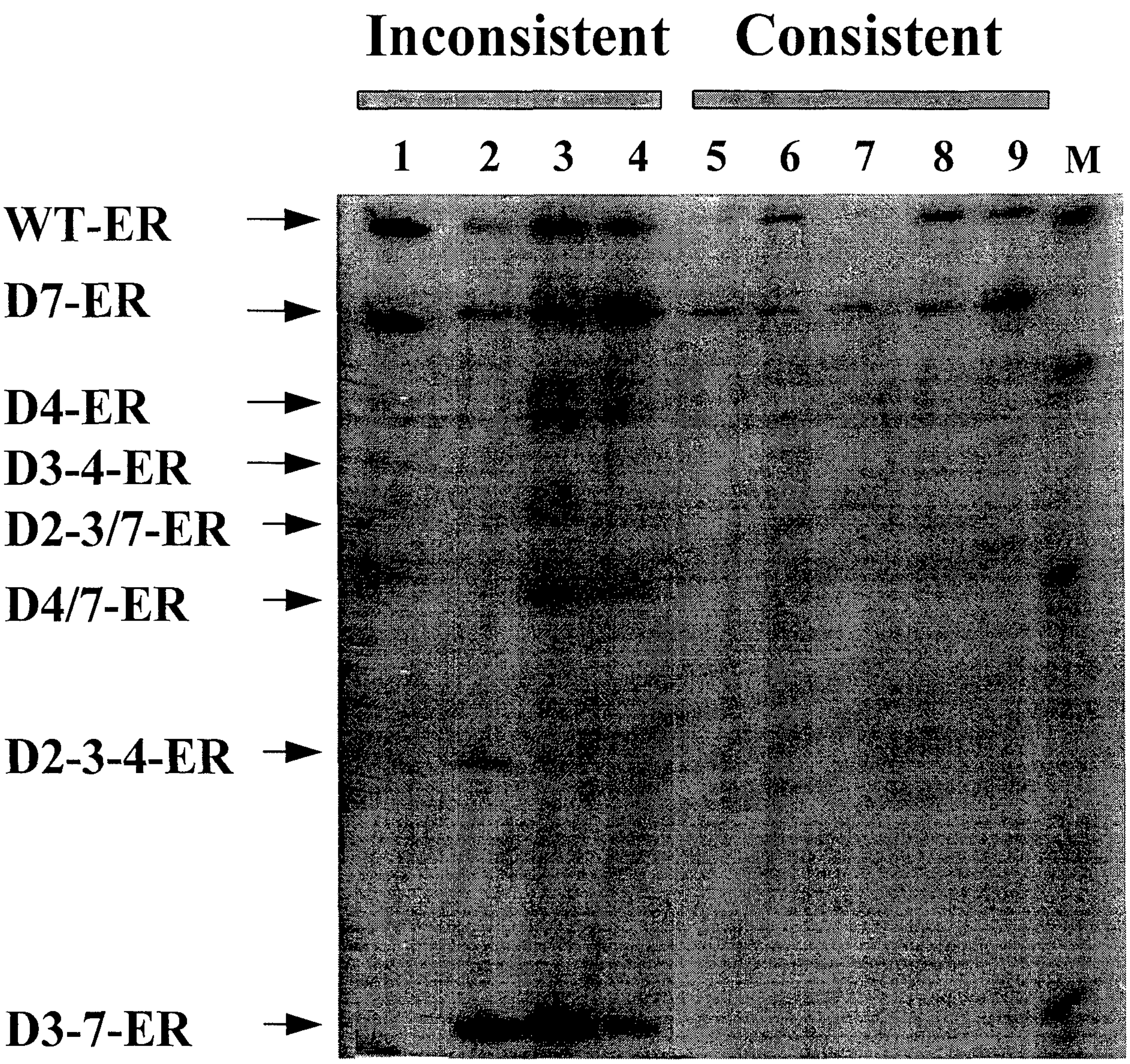
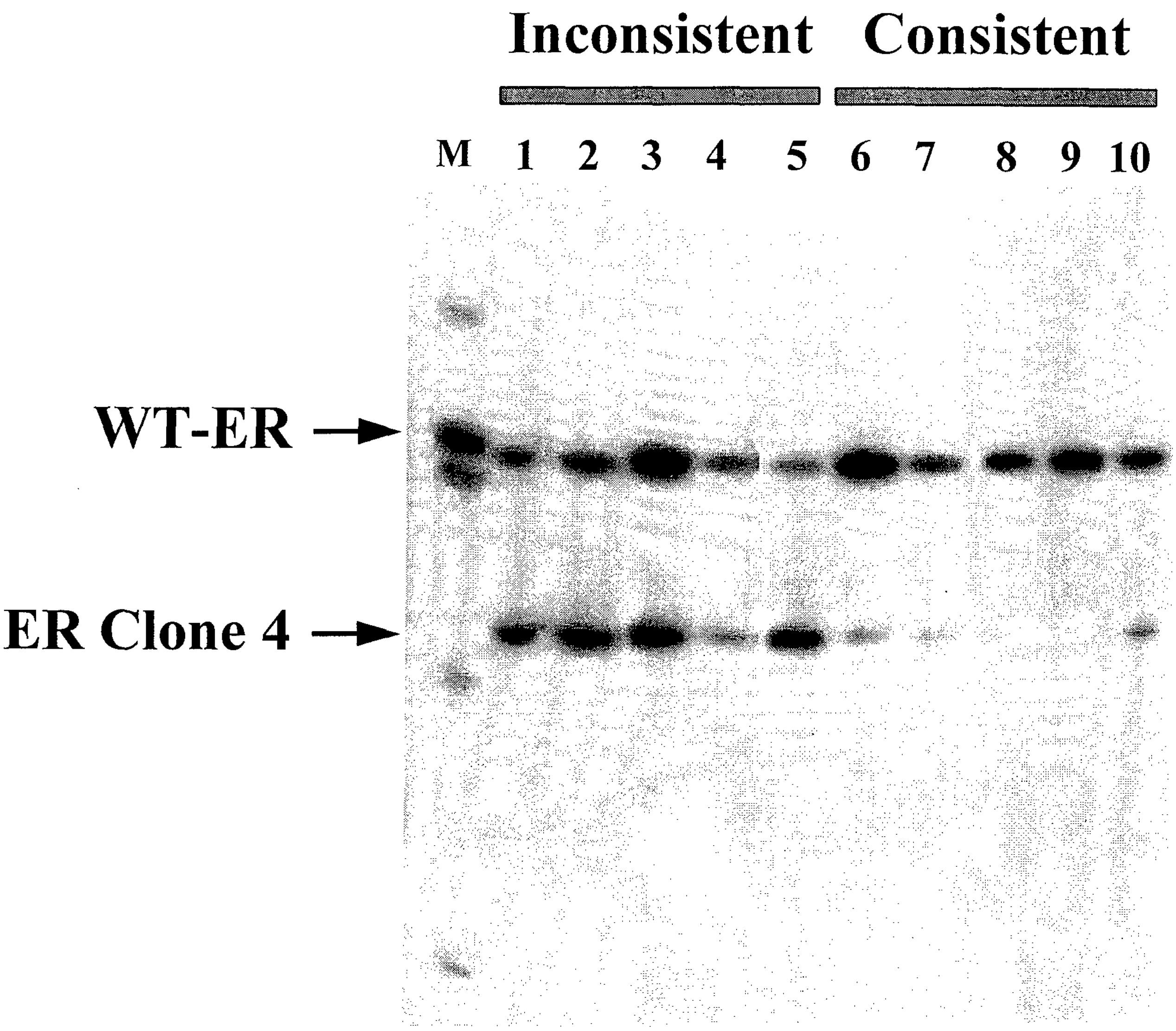
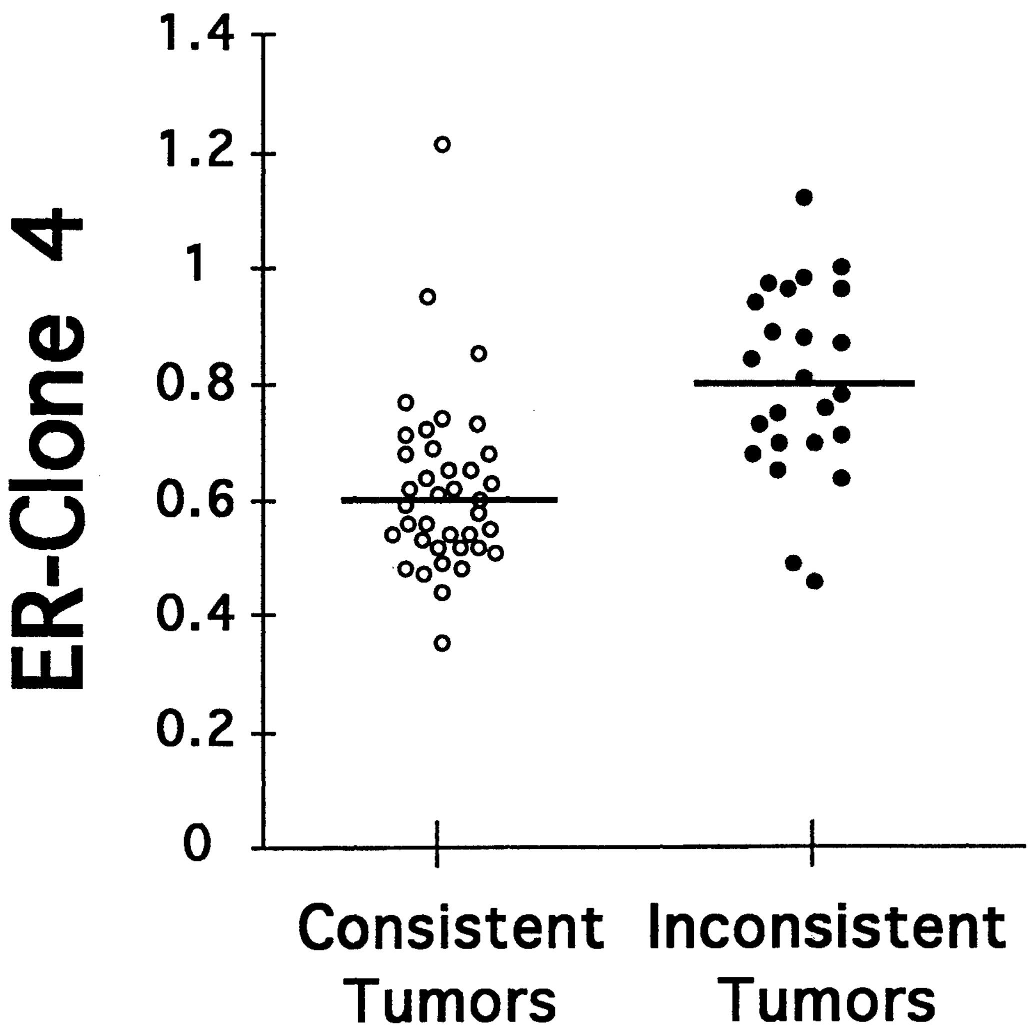


Figure 2. Chart showing the difference in ER level determined by immunohistochemical assay and H score with 1D5 or AER-311 antibody for 63 ER positive breast tumors. These are classified as 'Consistent Tumors' (H score difference < 50, 39 cases, white bars), or 'Inconsistent Tumors' (H core difference >50, 24 cases, black bars).


Inconsistent
Consistent
1
2
3
4
5
6
7
8
9
M
WT-ER
D7-ER
D4-ER
D3-4-ER
D2-3/7-ER
D4/7-ER
D2-3-4-ER
D3-7-ER


Inconsistent
Consistent
M 1 2 3 4 5 6 7 8 9 10
WT-ER
ER Clone 4



ER-Clone 4
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Consistent Tumors
Inconsistent Tumors

Table 1: Number of tumors expressing detectable ER variant in consistent and inconsistent tumors

Tumors	N	D7-ER	D4-ER	D3-4-ER	D4/7-ER	D2-3/7-ER	D2-3-4-ER	D-3-7-ER	ER V.^{OF}	ER V.^{IF}
Consistent	39	35	9	3	4	0	0	2	6	12
Inconsistent	24	22	6	2	2	2	3	8	10	8
P		>0.05	>0.05	>0.05	>0.05	>0.05	**<0.05**	**<0.01**	**<0.05**	>0.05

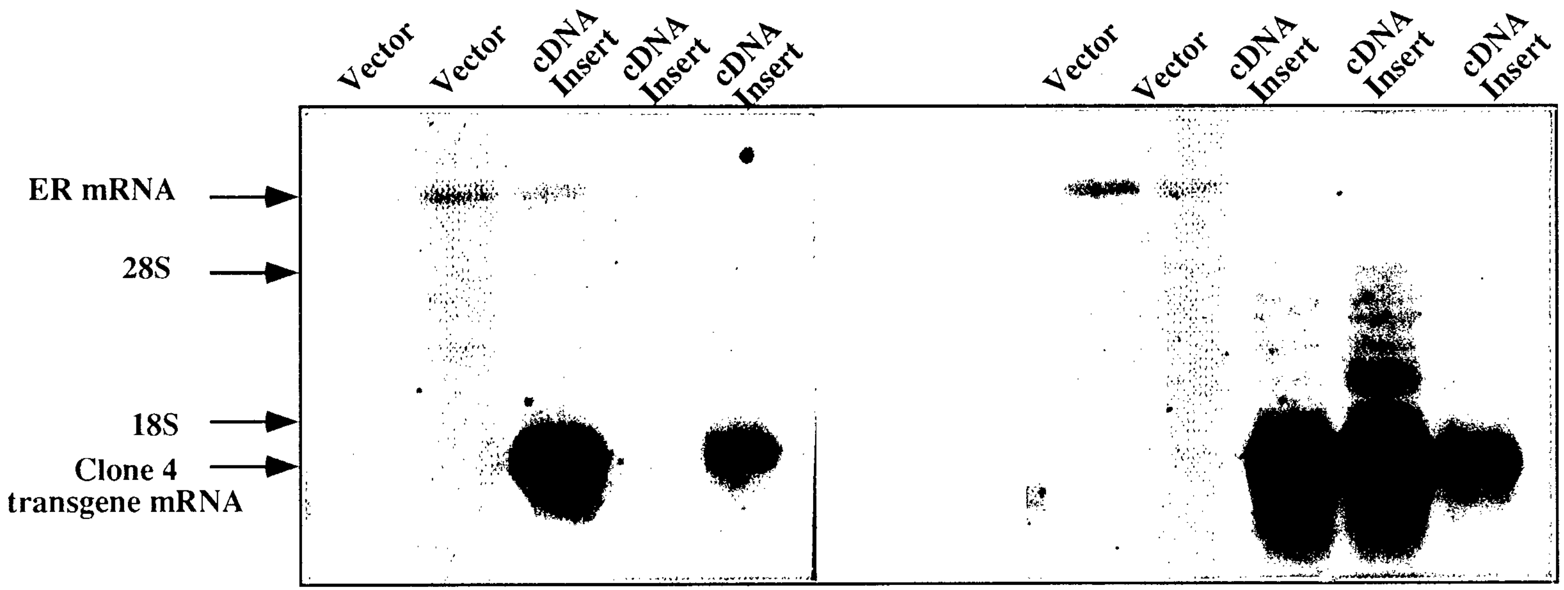
Chisquared analysis

ER V.^{OF}: out-of-frame exon-deleted ER variant mRNAs excluding D7-ER

ER V.^{IF}: in-frame exon-deleted ER variant mRNAs

APPENDIX 13

Murphy / Watson: MCF 7 Human Breast Cancer Cells Stably Transfected with Expression Vector Alone and Clone 4 Transgene Containing Vector.



Recd
1/11/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the attached Grants. Request the limited distribution statements for Accession Document Numbers listed be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at Judy.Pawlus@amedd.army.mil.

FOR THE COMMANDER:

Phylis Rinehart
PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

95-1-5015 AD-B222447

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